PULMONARY NEUTROPHIL RECRUITMENT, ACTIVATION AND CLEARANCE IN EQUINE CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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DECLARATION

This thesis was composed entirely by myself on the basis of my experimental work performed under the supervision of Professors Edwin R. Chilvers and Christopher Haslett in The Rayne Laboratory, Respiratory Medicine Unit, Department of Medicine and Drs. Bruce C. McGorum and Padraic M. Dixon in The Wellcome Trust Centre for Research in Comparative Respiratory Medicine, Department of Veterinary Clinical Studies, The University of Edinburgh.

Tim Brazil,
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ABSTRACT

Chronic obstructive pulmonary disease (COPD) in the horse is caused by exposure of susceptible animals to dust and aeroallergens in poorly saved hay and straw. It is characterized by recurrent episodes of airway obstruction in association with neutrophilic pulmonary inflammation. As neutrophils have the capacity to secrete a wide array of potentially histotoxic products such as reactive oxygen species, proteases and cationic proteins, these cells have been proposed to be key mediators of the lung injury observed in COPD. The functional status of neutrophils within both the circulating granulocyte pool and the airways of horses with COPD remains largely unexplored. Likewise, little is known of the kinetics of airspace neutrophil recruitment and clearance during and after an acute episode of COPD or indeed the subsequent fate of these cells.

I have demonstrated *in vitro* that isolated peripheral blood neutrophils may be primed by exposure to inflammatory mediators such as lipopolysaccharide, platelet activating factor and tumour necrosis factor-α. This results in enhanced respiratory burst activity upon subsequent exposure to secretagogue stimuli. Indeed, priming was found to be a necessary step in the induction of functional coupling of receptors for the bacterial peptide fMLP in equine neutrophils.

I have also demonstrated that purified peripheral blood neutrophils undergo apoptosis constitutively when aged *in vitro* and that the rate of apoptosis can be modulated by exposure to a range of pro-inflammatory mediators. In other models of neutrophilic inflammation, cells that have undergone apoptosis have been shown to be recognized by phagocytes such as inflammatory macrophages, engulfed and degraded without inciting an inflammatory response.

Acute COPD was induced in susceptible horses by exposure to a 5 h hay/straw challenge. The kinetics and function of airspace neutrophils harvested by bronchoalveolar lavage and peripheral blood neutrophils was monitored sequentially for 14 days after challenge. Hay/straw challenge primed both peripheral blood and airspace neutrophils for an enhanced secretagogue-induced respiratory burst. Significant degranulation of neutrophils occurred within the airspaces as evidenced

by a marked increase in neutrophil elastase in bronchoalveolar lavage fluid. As the pulmonary inflammation resolved, airspace neutrophils underwent apoptosis and were phagocytosed by alveolar macrophages *in vivo*. This work demonstrates significant upregulation of neutrophil function in horses with COPD and provides evidence of a pivotal role for neutrophil apoptosis in the resolution of the pulmonary neutrophil burden in this disease.

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DEDICATION

To my parents, Jane and Bernard Brazil, without whose support and sacrifice I would not have made it this far.

To two Veterinary Surgeons who continue to inspire me in the pursuit of excellence

Jack de Garis MRCVS

And

Richard Taylor BVM&S MRCVS 1961 - 1998

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ABBREVIATIONS

0.9% NaCl-T20	0.9% NaCl containing 0.05% Tween 20
α-1PI	α-1 proteinase inhibitor
ATP	Adenosine triphosphate
ARDS	Acute respiratory distress syndrome
AEC	3-amino-9-ethylcarbazole
(k)bp	(kilo)base pairs
BSA	Bovine serum albumin
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
COPD	Chronic obstructive pulmonary disease
CL	Chemiluminescence
CD	Cluster of differentiation
C5a	Complement fragment 5a
CR3	Complement receptor 3
DAB	3,3'-diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
DEX	Dexamethasone
EC_{50}	Concentration of a stimulus producing 50% of maximal response
ELISA	Enzyme-linked immunosorbent assay
ENE 2A	Equine Neutrophil Elastase 2A
ENE 2B	Equine Neutrophil Elastase 2B
FasL	Fas ligand
Fc	constant region of IgG
FITC	Fluorescein isothiocyanate
FACS	Fluorescence-activated cell sorter
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor

HBSS	Hank's balanced salt solution
HRPO	Horseradish peroxidase
HEPES	N-(2-hydroxymethyl) piperazine N'-2-ethanesulphonic acid
h	hour(s)
h(e)r	human (equine) recombinant
-OH	hydroxyl group
Ig	Immunoglobulin
IC_{50}	Concentration of a stimulus producing 50% of its maximal inhibitory response
ICAM	Intercellular adhesion molecule
IL-	Interleukin
i/v	intravenously
L	Ligand (e.g. FasL)
LT	Leukotriene
LPS	Lipopolysaccharide
Luci-DCL	Lucigenin-dependent chemiluminescence
Lum-DCL	Luminol-dependent chemiluminescence
MMP-	Matrix metalloproteinase
mRNA	messenger RNA
MPBS	5% dried skimmed milk in PBS
min	minutes
Mab	Monoclonal antibody
MPO	Myeloperoxidase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NRS	Normal rabbit serum
p38-MAPK	p38-mitogen-activated protein kinase
PMA	Phorbol 12-myristate 13-acetate
PBS	phosphate buffered saline with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$
PBS w/o	Phosphate buffered saline without 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$

PAF	Platelet activating factor
PECAM	Platelet-endothelial cell adhesion molecule
PPP	Platelet poor plasma
PKC	Protein Kinase C
PELF	Pulmonary epithelial lining fluid
ROS	Reactive oxygen species
RAO	Recurrent airway obstruction
RLU	Relative Light Units
RNA	Ribonucleic acid
SEM	Scanning electron microscopy
± SEM	plus and minus standard error of the mean
TESPA	3-aminopropyltriethoxysilane
TUNEL	Terminal deoxynucleotide transferase mediated UTP Nick End-Labelling
TMB	3,3',5,5'-tetramethylbenzidine
TEM	Transmission electron microscopy
Tris	Tris (hydroxymethyl) aminomethane-Cl
TNF-α	Tumour necrosis factor-α
TNFR	Tumour necrosis factor-α receptor
U	Units
ZAP	Zymosan activated plasma
ZAS	Zymosan activated serum

All other abbreviations represent standard SI units

CHAPTER 1

INTRODUCTION

1.1 EQUINE CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Since man first domesticated the horse, moving it from its traditional range habitat, an association has been recognized between the development of chronic pulmonary disease and the feeding of dried forage in dusty stabling conditions. Indeed this is not solely an affliction of the modern-day domesticated horse; a similar disorder having been observed in zebras and Przewalskii wild horses when confined in zoos (Beech, 1991). The syndrome of coughing, nasal discharge, exercise intolerance and respiratory embarrassment has been known to both the equine fraternity and the veterinary profession by a succession of pseudonyms; "hay sickness", "broken wind", "alveolar emphysema", "heaves", "chronic bronchiolitis", "chronic small airway disease", "chronic obstructive pulmonary disease" (COPD) and "recurrent airway obstruction" (RAO). Since Sasse (1971) first introduced the term COPD to describe horses with this syndrome, it has remained in common usage in Europe and will be used throughout this thesis.

Given the similarity of the presenting clinical picture in many chronic equine lung diseases and the necessity for comparison of data with other laboratories it is important to clearly define, at the outset, the criteria for the diagnosis of COPD in the horses studied in these investigations.

Horses were defined as suffering from COPD if they had a history (> 6 months) of recurrent episodes of pulmonary disease, consistently induced by exposure to hay and/or straw and characterized by clinical signs of airway obstruction, disruption of pulmonary gas exchange (manifested by arterial hypoxaemia) and the presence of increased numbers of bronchoalveolar neutrophils (> 5% neutrophils in bronchoalveolar lavage fluid, BALF). Moreover, horses entered complete remission from disease following their removal from a provocative environment containing hay and/or straw to pasture or to a "controlled" environment (q.v.) in a well-ventilated

stable, bedded on wood shavings and fed dust-free haylage. Horses which had a history of developing obstructive pulmonary disease whilst at pasture during the summer months, so-called summer pasture associated obstructive pulmonary disease (Dixon and McGorum, 1990) were excluded from the study whether they satisfied the preceding criteria or not.

1.1.1 EPIDEMIOLOGY OF EQUINE COPD

In the Northern Hemisphere, COPD is the most commonly recognized chronic pulmonary disease of the horse (McPherson and Thomson, 1983) and in some northern European countries is thought to be the primary cause of premature loss through disease (Gerber, 1973). A recent retrospective study of 300 clinical cases of pulmonary disease examined at The University of Edinburgh identified 54.8% of horses as suffering from COPD (Dixon *et al.*, 1995b). The recorded incidence of COPD, although likely to be influenced by both local management practices and the diagnostic criteria employed, has been reported to be as high as 54% and even 79% amongst some horse populations in Switzerland (Bracher *et al.*, 1991) and the Netherlands (Sasse *et al.*, 1985), respectively.
COPD is generally recognized in middle-aged to older horses and the incidence increases with age (Naylor *et al.*, 1992; Dixon *et al.*, 1995b; Ainsworth, 1999). No apparent sex or breed predilection has been observed (McPherson and Thomson, 1983; Beech, 1991).

1.1.2 CLINICAL MANIFESTATIONS OF DISEASE

The presenting clinical history and signs in COPD-affected horses can be highly variable depending upon the severity and stage of disease and the level of exposure of susceptible horses to airborne antigens and stable dust. Moreover, both the diagnostic criteria and the sensitivity and specificity of diagnostic aids employed by an individual clinician will affect the diagnosis rate. When in complete remission

COPD-susceptible horses are clinically normal and indistinguishable from healthy animals.

Coughing, mucopurulent nasal discharge, decreased exercise tolerance and prolonged post-exercise dyspnoea may be detected in mildly affected animals (McPherson *et al.*, 1978; Beech, 1991; Naylor *et al.*, 1992; Dixon *et al.*, 1995b). More severely affected cases exhibit tachypnoea and increasing degrees of dyspnoea ("heaves") at rest, often with a markedly increased expiratory effort necessitating recruitment of the abdominal muscles (so-called double expiratory effort) which in longstanding severely affected animals results in hypertrophy of the external abdominal oblique muscles producing a characteristic "heave line". Such animals may also show nostril flaring and anal pumping with breathing (Beech, 1991; Naylor *et al.*, 1992).

Abnormal respiratory sounds are detectable at rest on auscultation of the trachea at the thoracic inlet (discontinuous fluid sounds) and the lung fields (inspiratory and expiratory crackles and wheezes) in 50-70% of affected horses and such sounds are more consistently detected (90%) during induced (by temporary nasal occlusion or with a rebreathing bag) hyperpnoea (Naylor *et al.*, 1992; Dixon *et al.*, 1995b).

1.1.3 AETIOLOGY OF EQUINE COPD

COPD is an environmental lung disease. The development of pulmonary hypersensitivity to inhaled material can almost be regarded as an occupational hazard of the housed, domesticated equid. Indeed movement of affected animals to a "clean air" environment either at pasture or stabled in a "controlled" environment (q.v.) results in complete clinical remission (Thomson and McPherson, 1984; Derksen *et al.*, 1985a; Derksen *et al.*, 1985b; McGorum *et al.*, 1993d). Studies of stable aerobiology have identified a plethora of materials that could be inhaled, such as bacteria and their component endotoxins, fungal components (including spores, hyphae and mycotoxins), noxious gases (e.g. ammonia and hydrogen sulphide), forage mites, animal-derived material (e.g. hair and faeces) and plant fragments (Clarke, 1987; Woods *et al.*, 1993; McGorum *et al.*, 1998). Spores of the approximately 70 species of moulds and actinomycetes that are the main constituents

of hay dust are of sufficiently small diameter (<5μm) to be respirable into the distal small airways and alveoli (Clarke, 1987).

Specific antigen inhalation challenges (McPherson *et al.*, 1979; Derksen *et al.*, 1988; McGorum *et al.*, 1993d), intradermal antigen testing (Eyre, 1972; Halliwell *et al.*, 1979; McPherson *et al.*, 1979; Evans *et al.*, 1992), detection of serum precipitins (Madelin *et al.*, 1991), local pulmonary production of specific IgE and IgG (Halliwell *et al.*, 1993; Schmallenbach *et al.*, 1998) and positive Prausnitz-Küstner passive cutaneous anaphylactic reactions and positive *in vitro* Schultz-Dale anaphylactic reactions (Eyre, 1972) suggest that equine COPD is a pulmonary hypersensitivity to inhaled mould antigens. Inhalation challenge with extracts of the fungus *Aspergillus fumigatus* or the thermophilic actinomycete *Faenia rectivirgula* induced pulmonary dysfunction and BALF neutrophilia in COPD-susceptible horses but not control (non-COPD) animals (McPherson *et al.*, 1979; Derksen *et al.*, 1988; McGorum *et al.*, 1993d). Although the results of intradermal antigen testing and measurement of serum precipitins to mould antigens did not reliably differentiate COPD-susceptible and non-hypersensitive animals, COPD-affected horses did have a greater percentage of positive skin reactions (Halliwell *et al.*, 1979; Evans *et al.*, 1992; McGorum *et al.*, 1993a).

Despite the aforementioned evidence specifically implicating hypersensitivity to certain mould spores (Derksen *et al.*, 1988; McGorum *et al.*, 1993d), the exact range of antigens involved remains unknown. Moreover, the pulmonary response to inhalation of individual mould extracts (e.g. *Aspergillus fumigatus*, *Faenia rectivirgula*) was substantially less than following "natural" hay/straw challenge (McGorum *et al.*, 1993d), suggesting that other inhaled agents contribute to the "natural" disease. Indeed, other important components of stable dust such as endotoxin (McGorum *et al.*, 1998) may act as non-specific irritants or toxins (Clapp *et al.*, 1994; Pirie *et al.*, 1998) to enhance the response to mould antigens.

The stimuli for development of pulmonary hypersensitivity are unknown but preceding viral infection (Gerber, 1973; McPherson and Lawson, 1974; Halliwell *et al.*, 1993) and an underlying genetic predisposition, as inferred from progeny testing of two COPD-susceptible stallions (Marti *et al.*, 1991), have been suggested.

1.1.4 PATHOLOGY OF EQUINE COPD

Although a number of post-mortem studies have reported the gross, histopathological and ultrastructural changes in mild, moderate and severe cases with active disease, little comparative data from clearly defined asymptomatic, clinically diseased and horses entering clinical remission is available.

Grossly, the lungs of asymptomatic and mildly affected animals appear normal. In severely affected cases the lungs may fail to collapse upon opening the thorax due to air trapping in the airways (Nicholls, 1978). This finding led early investigators to erroneously conclude that these animals had emphysema (Gillespie and Tyler, 1969; Gerber, 1973).

Pathological changes in the large, conducting airways are restricted to focal areas of goblet and epithelial cell hyperplasia with loss and malformation of ciliated cells. These bronchial changes correlated poorly with clinical disease and were considered to be non-specific and of minor importance (Kaup *et al.*, 1990b).

Pathology of the small airways and alveoli, although focal, is widely distributed throughout the lungs and correlates well with the severity of clinical disease (Kaup *et al.*, 1990a; Nyman *et al.*, 1991; Naylor *et al.*, 1992). The consistent feature is that of a neutrophilic bronchiolitis with an inflammatory exudate and excess mucus in airway lumina and associated reactive changes in adjacent alveoli (Nicholls, 1978; Winder and Von Fellenberg, 1988; Kaup *et al.*, 1990a). Other bronchiolar features include epithelial and goblet cell hyperplasia and metaplasia, Clara cell degranulation and degeneration, peribronchiolar infiltration with monocytes, lymphocytes, mast cells and plasma cells and fibrosis, airway smooth muscle hypertrophy and perivascular plasma-lymphocytic cuffing (Nicholls, 1978; Winder and Von Fellenberg, 1988; Kaup *et al.*, 1990a; Nyman *et al.*, 1991). Small airway morphometry in acutely diseased animals confirmed increases in airway epithelial, smooth muscle and total wall thickness (Broadstone *et al.*, 1997). The above changes lead to bronchiolar narrowing and mucus plugging of the small airways. Alveolar changes including increased numbers of Kohn's pores, necrosis of type I and transformation of type II epithelial cells, alveolar fibrosis and alveolar

hyperinflation are focal and inconsistent, even in severely affected cases. Alveolar emphysema is rare (Nicholls, 1978; Kaup *et al.*, 1990a; Nyman *et al.*, 1991).
Two recent *in vivo* studies provide preliminary insights into the functional pathology of airway cells and extracellular matrix during episodes of equine COPD. Firstly, Votion and Colleagues (1999) reported increased alveolar clearance consistent with alveolar epithelial cell injury following exposure of COPD-susceptible horses to a hay/straw challenge. Secondly, a 7-fold increase in the concentration of the extracellular matrix polysaccharide, hyaluronate, in the tracheal lavage fluid of COPD-affected horses in comparison to healthy controls was hypothesised to reflect pathological remodelling of peribronchiolar connective tissue (Tulamo and Maisi, 1997).
The overall picture is one of limited irreversible structural lung pathology. This is supported by the work of Naylor and colleagues (1992), who reported that the severity of clinical signs was best correlated with the degree of bronchiolar neutrophil infiltration and goblet cell metaplasia (potentially reversible features) in percutaneous lung biopsies. The absence of significant permanent lung pathology is also indicated by the recognition that, with appropriate management, many affected horses may achieve full clinical remission (Thomson and McPherson, 1984; Dixon *et al.*, 1995d).

1.1.5 PATHOGENESIS OF EQUINE COPD

The increased concentrations of mould-specific immunoglobulins (Section 1.1.3) and numbers of B lymphocytes (McGorum *et al.*, 1993b; Watson *et al.*, 1997; Rush *et al.*, 1998a) in BALF, increased levels of both free and lung parenchymal cell-associated immunoreactive IgA and IgG(Fc) (Winder and Von Fellenberg, 1988) and localised alveolar mast cell infiltration (Kaup *et al.*, 1990a) in COPD-susceptible horses support an immunological or allergic basis to the pulmonary hypersensitivity. However, the absence of early signs of airway obstruction and the presence of a delayed (3-5 h) inflammatory response following hay/straw challenge are suggestive of a delayed, probably Type III, hypersensitivity response (McGorum *et al.*, 1993c; Robinson *et al.*, 1996).

The two cardinal and complimentary features of equine COPD are airway inflammation and bronchoconstriction (Derksen, 1993; Robinson *et al.*, 1996). Both responses are likely to be initiated and interconnected by a complex array of inflammatory mediators. The roles of inflammatory cells and mediators and their associations with airway smooth muscle function in equine COPD will be reviewed.

1.1.5.1 Inflammatory cells

Increased neutrophil numbers in the tracheal secretions and/or BALF of affected horses is the hallmark of the cellular inflammatory response in both clinical and experimental studies of equine COPD (Nuytten *et al.*, 1983; Derksen *et al.*, 1985b; Bracher *et al.*, 1991;Vrins *et al.*, 1991; Naylor *et al.*, 1992; McGorum *et al.*, 1993d; Dixon *et al.*, 1995c). The structure and function of neutrophils and their potential to cause host tissue injury are discussed later. The kinetics and function of airspace neutrophils in lung inflammation are reviewed in detail in Chapter 6.

The initiation of pulmonary inflammation in COPD-susceptible horses represents a specific immune-mediated response to inhaled antigens rather than a non-specific response to airborne irritants or toxins (McGorum *et al.*, 1993d). Subsequent neutrophil recruitment is probably orchestrated by chemoattractant cytokines, eicosanoids and other lipid mediators (e.g. platelet activating factor, PAF) derived from airway epithelial cells and resident lymphocytes, macrophages and mast cells (Shelhamer *et al.*, 1995; Lukacs *et al.*, 1996).

The Th2 cytokine phenotype subset of CD4+ T-helper lymphocytes appears to mediate and maintain airway inflammation in human atopic asthma (Lukacs *et al.*, 1996). CD4+ T-cells are also recruited to the lungs of COPD-susceptible horses following exposure to hay and straw (McGorum *et al.*, 1993b; Rush *et al.*, 1998a) and thus may mediate neutrophil maturation, recruitment and function in this disease. The kinetics and cytokine profiles of pulmonary lymphocyte subsets in equine COPD warrants urgent attention.

Although interest has focussed on airspace neutrophils as the key mediators of pulmonary inflammation in COPD, resident alveolar macrophages may be critically important throughout the genesis, amplification and mediation of lung injury and not least in the resolution of this inflammation. Alveolar macrophages may recognize

and phagocytose inhaled particles by both non-specific and specific immune mechanisms via Fc, complement and IgE receptors (Rankin, 1989). Of note, mould spores can directly stimulate pro-inflammatory chemokine (e.g. Interleukin (IL)-8, macrophage inflammatory protein 1, MIP-1 and tumour necrosis factor-α, TNF-α) synthesis and release (Rankin, 1989; Shahan *et al.*, 1998) and superoxide anion generation (Shahan *et al.*, 1994) in rat alveolar macrophages. Stimulation of equine alveolar macrophages with lipopolysaccharide (LPS) induces synthesis of the potent neutrophil chemoattractants IL-8 and MIP-1 (Franchini *et al.*, 1998). Equine alveolar macrophages produce procoagulant activity *in vitro* (Grunig *et al.*, 1988), whose levels in tracheal secretions correlated well with the severity of chronic lung disease (Grunig *et al.*, 1988). Furthermore, secretion of reactive oxygen species (ROS) and proteolytic enzymes by macrophages can induce both direct lung cell injury and mucus hypersecretion (Rankin, 1989; Sibille and Reynolds, 1990; Pittet *et al.*, 1997). The role of alveolar macrophages in the resolution of lung inflammation is discussed in detail in Chapter 6.

Mast cells may play a role in equine COPD through the release of cytokines (Shelhamer *et al.*, 1995) and histamine (Hare *et al.*, 1999). Although both peripheral blood basophils (Dirscherl *et al.*, 1993) and pulmonary mast cells (Hare *et al.*, 1999) from COPD-susceptible horses release greater amount of histamine than cells from normal animals when exposed to mould extracts *in vitro*, histamine levels are only increased in BALF 5 h after hay/straw challenge (McGorum *et al.*, 1993c). As pulmonary neutrophil recruitment and airway obstruction are already well established by this time (Fairbairn *et al.*, 1993), the pathogenetic role of mast cell products remains unclear.

The importance of bronchial epithelial cell-derived cytokines (e.g. IL-8 and granulocyte-macrophage colony stimulating factor, GM-CSF) in the recruitment (Shelhamer *et al.*, 1995) and functional modulation (Cox *et al.*, 1992) of airspace neutrophils has received little attention in the horse and is likely to have been greatly underestimated.

In contrast to allergic asthma in humans (Lukacs *et al.*, 1996), eosinophils appear to have no role in equine COPD (McGorum *et al.*, 1993d; Robinson *et al.*, 1996).

1.1.5.2 Inflammatory mediators

Largely due to a lack of well-characterized immunological reagents, the mediators involved in equine COPD have to date received little attention. Notable exceptions are arachidonic acid-derived lipid mediators because commercially available human radioimmunoassay kits for many are highly cross-reactive with the equivalent equine molecules (Gray *et al.*, 1989; Watson *et al.*, 1992).

Increased concentrations of prostaglandin E_2 and $F_{2\alpha}$ in BALF (Watson *et al.*, 1992) and thromboxane B_2 (Gray *et al.*, 1989) and 15-hydroxyeicosatetranoic acid (15-HETE) (Gray *et al.*, 1992a) in plasma of COPD-susceptible horses following hay/straw challenge indicates activation of the arachidonic acid cascade. However, despite abrogating the thromboxane B_2 response, the failure of a cyclooxygenase inhibitor to reduce airway obstruction following challenge suggested that cyclo-oxygenase products were of minor importance in equine COPD (Gray *et al.*, 1989). Although BALF levels of the anti-inflammatory mediators prostaglandin E_2 and 15-HETE are increased after challenge, local airway mucosal production of prostaglandin E_2 is significantly reduced (Gray *et al.*, 1992b), suggesting that a lack of inhibitory prostanoid function may facilitate airway inflammation.

The cysteinyl leukotrienes (LTs), LTC_4, LTD_4 and LTE_4 contract airway smooth muscle, induce vasodilatation and mucus secretion and increase vascular permeability potentially facilitating leucocyte migration. Leukotriene B_4 potently stimulates neutrophil chemotaxis (Busse, 1998). Some *in vivo* evidence suggests involvement of these products of the 5-lipoxygenase pathway of arachidonic acid metabolism in equine COPD. Doucet *et al.* (1991) found increased levels of LTE_4 in urine from horses with COPD, but concentrations of LTB_4 and C_4 in BALF were no different to control animals (Watson *et al.*, 1992). Inhalation of LTD_4 and B_4 in normal horses caused bronchoconstriction and pulmonary neutrophil recruitment, respectively, but the responses of asymptomatic COPD-susceptible horses were highly variable (Marr *et al.*, 1998b). However, pre-treatment with a 5-lipoxygenase inhibitor did reduce airway obstruction in 2 of 6 COPD-susceptible horses after hay/straw challenge (Marr *et al.*, 1998a).

Although intravenously administered PAF causes transitory lung dysfunction and pulmonary neutrophil retention in both COPD-susceptible and normal horses

(Fairbairn *et al.*, 1996), pre-treatment with a PAF-receptor antagonist had no effect on either neutrophil recruitment or lung dysfunction when COPD-susceptible animals were challenged (Marr *et al.*, 1996b).

As discussed in Section 1.1.5.1, the role of histamine in the pulmonary response to hay/straw challenge is unclear, but the poor response of clinical COPD cases to anti-histamine therapy would suggest that it is of minor significance (Beech, 1991).

The local pulmonary cytokine network is likely to play a central role in the pathogenesis of airway inflammation in equine COPD (Shelhamer *et al.*, 1995; Lukacs *et al.*, 1996) but the current paucity of such reagents for the horse have hampered progress in this field.

Respiratory tract secretions from COPD-affected horses contain elevated levels of proteolytic activity, especially collagenase (matrix metalloproteinase, MMP-8) and gelatinase (MMP-2 and MMP-9) activity (Koivunen *et al.*, 1996; Koivunen *et al.*, 1997a; Koivunen *et al.*, 1997b). These are most likely of neutrophil and/or macrophage origin and may have a causal relationship with mucus hypersecretion, lung injury and the amplification of the airway inflammatory response (Kumagai *et al.*, 1999). Neutrophil granule enzymes, including MMP's are discussed in Section 1.2.2.

1.1.5.3 Bronchoconstriction

The pulmonary dysfunction detected in COPD-affected horses (see Section 1.1.6) is rapidly relieved but not eliminated by administration of bronchodilators (Broadstone *et al.*, 1988; Robinson *et al.*, 1993; Derksen *et al.*, 1999), suggesting that bronchospasm is a major component of the airway obstruction. The innervation of the equine lung and the complex mechanisms involved in regulation of airway smooth muscle tone in equine COPD have been extensively reviewed (Robinson *et al.*, 1996; Robinson, 1997b); hence only a brief discussion of the interactions between airway inflammation and bronchspasm that are pertinent to this study will be given.

The consistent bronchodilation afforded in affected animals by the anticholinergic drugs atropine and ipratropium bromide (Broadstone *et al.*, 1988; Robinson *et al.*,

1993) indicate that bronchspasm is mediated in large part by activation of muscarinic, M_3 receptors by acetylcholine.

As well as acetylcholine release from parasympathetic nerves in response to inhaled irritants and inflammatory mediators, airway smooth muscle contraction may also be enhanced by local and central release of neuropeptides following stimulation of excitatory nonadrenergic-noncholinergic sensory nerves. Of note, in laboratory animals, prolonged bronchoconstriction *per se*, possibly through local neuropeptide release, can also stimulate mucus secretion and neutrophil chemotaxis (Barnes, 1999).

Inhibitory sympathetic nerves release norepinephrine, activating β_2 adrenoreceptors to mediate bronchodilation. Although these receptors are present on airway smooth muscle throughout the respiratory tract, in the lower airways, smooth muscle relaxation only occurs in response to circulating norepinephrine. The inhibitory effect of β_2-adrenoreceptor stimulation is employed by β_2 agonist bronchodilators. The inhibitory nonadrenergic-noncholinergic system acts via nitric oxide release to inhibit airway smooth muscle contraction; critically function of this inhibitory system is consistently absent in COPD-affected horses (Yu *et al.*, 1994). Whether this reflects an underlying feature of COPD-susceptible animals or inactivation of the neurotransmitter nitric oxide by local inflammatory mediators is unknown. In the trachealis muscle at least, physiological concentrations of the neutrophil secretory product hydrogen peroxide (H_20_2), did not inactivate inhibitory nonadrenergic-noncholinergic function *in vitro* (Olszewski *et al.*, 1995).

A number of the inflammatory mediators released in equine COPD (Section 1.1.5.2) can induce or enhance bronchspasm. *In vitro*, histamine, leukotriene D_4 and serotonin stimulation and blockade of cyclooxygenase (and by inference blockade of potentially inhibitory prostaglandin synthesis) augment cholinergic contractile responses of equine small airways (Olszewski *et al.*, 1997; Olszewski *et al.*, 1999). An interesting finding from these *in vitro* studies was a high level of individual variability in the contractile response to LTD_4 of small airways isolated from COPD-affected horses (Olszewski *et al.*, 1999). In light of the variable response of COPD-susceptible horses to inhaled LTD_4 *in vivo* (Marr *et al.*, 1998b), these data suggest

significant heterogeneity in the relationship between inflammatory mediators and bronchospasm.

The role of neutrophil secretory products in the bronchospastic response is not entirely clear. *In vitro*, high concentrations of H_2O_2 (1 mM) can stimulate contraction of isolated trachealis muscle (an upper airway muscle) and lower concentrations (100 μM) modulate neurotransmission and muscarinic receptor function and induce cyclooxygenase activity (Olszewski *et al.*, 1995). However, perhaps more critically, the cholinergic tone of small airways (the focus of the inflammatory response in COPD) harvested from both COPD-affected and control horses was unaffected by co-incubation with zymosan-activated peripheral blood neutrophils, suggesting that neutrophil secretory products are not involved in the pathogenesis of bronchospasm (Olszewski *et al.*, 1999).

A further important feature of the altered bronchomotor function in equine COPD is the development of airway hyperresponsiveness. Enhanced sensitivity to non-specific bronchospastic agents such as histamine, methacholine and citric acid has been reported in affected animals (Derksen *et al.*, 1985a; Armstrong *et al.*, 1986; Fairbairn *et al.*, 1993). Even after 7 h of exposure to hay and straw, airway hyperresponsiveness persists in susceptible animals for up to 3 days (Fairbairn *et al.*, 1993). This may have significant implications for the management of clinical COPD cases, wherein even transitory exposure to antigen can increase their susceptibility to episodes of bronchospasm for prolonged periods.

Bearing in mind that the calibre of an airway varies as a factor of its radius to the fourth power, the airway obstruction induced by bronchospasm will be exacerbated if airway calibre is already diminished due to inflammation-driven airway wall thickening

1.1.6 PATHOPHYSIOLOGY OF EQUINE COPD

The combined effects of airway inflammation, bronchospasm and increased mucus secretion in affected horses lead to diffuse airway obstruction and the dyspnoea detected clinically. Widespread obstruction of small airways is readily detectable in severe cases by pulmonary mechanics testing where large increases in pulmonary

resistance with a concomitant reduction in dynamic compliance are detected. The horse responds to airway obstruction by increasing its respiratory effort, detectable as an increase in the maximal change in intrapleural pressure. Diffuse airway obstruction also results in abnormal distribution of ventilation causing an increase in dead space and significant ventilation-perfusion mismatching (Nyman *et al.*, 1991), the net effect of which is impaired gas exchange and arterial hypoxaemia (reduced PaO_2) (West, 1992). However, hypercapnia (increased $PaCO_2$) is not a feature, suggesting that hypoventilation does not occur in spite of the obvious increase in respiratory effort that affected animals show. Hypoxaemia stimulates respiratory drive, increasing respiratory rate and minute volume although tidal volume is unchanged. To maintain tidal volume in the face of increased respiratory frequency, affected horses change their breathing strategy to accommodate greatly increased inspiratory and especially early expiratory flow rates (Robinson *et al.*, 1999).

Many of the alterations in pulmonary function described above have been documented in experimental hay/straw challenge studies in which affected animals become severely dyspnoeic (Derksen *et al.*, 1985b; McGorum *et al.*, 1993d; Vandenput *et al.*, 1998; Robinson *et al.*, 1999). Evaluation of pulmonary mechanics is relatively insensitive to subtle changes in pulmonary function (mildly and moderately affected horses) and difficult to perform routinely in clinical practice. However, clinical assessment of the degree of dyspnoea correlates well with both the degree of ventilation-perfusion mismatching (Nyman *et al.*, 1991) and the associated maximal change in intrapleural pressure (Dixon *et al.*, 1995c).

The importance of inflammation and mucus plugging of small airways in the development of lung dysfunction is clearly suggested by the failure of bronchodilator therapy to return pulmonary resistance to baseline values in experimentally induced COPD. Indeed, the degree of bronchiolar neutrophil infiltration and goblet cell metaplasia in biopsy material (Naylor *et al.*, 1992) and epithelial hyperplasia at necropsy (Nyman *et al.*, 1991) correlated with the severity of clinical signs and ventilation-perfusion mismatching respectively, suggesting that these features have significant effects on gas exchange.

Mucus hypersecretion may in fact be the rate-limiting step in the resolution of airway obstruction. Epithelial metaplasia and loss of cilia (Section 1.1.4) will reduce

mucociliary clearance rates and preliminary data suggest that excessive production of airway mucins may persist long after the resolution of airspace neutrophilia and overt lung dysfunction (Jeffcoat *et al.*, 1998).

Assessment of alveolar epithelial cell permeability by scintigraphic evaluation of the alveolar clearance of ^{99m}Technetium-labelled diethylene triamine penta-acetate (Votion *et al.*, 1998) has clearly demonstrated markedly increased epithelial permeability in the caudal lung fields of COPD-susceptible horses exposed to hay and straw (Votion *et al.*, 1999). This disruption of normal alveolar epithelial cell physiology is likely to have significant consequences for gas exchange. Two important conclusions can be drawn from this work. Firstly, the importance of assessing functional airway pathology rather than relying on the less sensitive structural assessment of biopsy or necropsy material. Secondly, this technique detected sub-clinical lung injury in horses housed in a suboptimal "controlled " environment (Votion *et al.*, 1999). These findings are consistent with the elevated concentrations of total protein and albumin in the pulmonary epithelial lining fluid (PELF) of COPD-affected horses in contrast to healthy controls (Milne *et al.*, 1994). These findings may be highly pertinent to the evaluation of the role of neutrophils in the pathogenesis of lung injury in equine COPD.

1.1.7 CLINICAL AND LABORATORY DIAGNOSIS OF EQUINE COPD

In many clinical cases of COPD a tentative diagnosis can be based upon a typical history and clinical examination findings as outlined previously. However, clinical findings alone are not specific for equine COPD and pulmonary disease may only be readily detected in severely affected animals (McPherson *et al.*, 1978; Dixon *et al.*, 1995b).

Whilst pulmonary function testing can confirm the presence of airway obstruction, it remains a relatively insensitive, non-specific and time consuming technique for routine evaluation (Viel, 1983; Dixon *et al.*, 1995a). However, measurement of pulmonary mechanics in combination with assessment of airway responsiveness to inhaled methacholine may be useful in the detection of subclinically affected animals housed in an inadequately managed environment (Vandenput *et al.*, 1998).

Measurement of arterial blood gas tensions and pH may be useful to identify or confirm abnormal gas exchange in affected animals. However, the diagnostic criterion of a PaO_2 below 85mmHg (temperature corrected) for COPD-affected horses suggested by McPherson *et al.* (1978) is likely to exclude many less severely affected animals; approximately 30% of the cases reported by Dixon *et al.* (1995c). Assessment of the distribution of ventilation by the nitrogen washout test (Viel, 1983; Robinson *et al.*, 1996) and alveolar epithelial cell permeability by scintigraphic evaluation of alveolar clearance (Votion *et al.*, 1998; Votion *et al.*, 1999) are both very sensitive measures of pulmonary function. Both techniques can detect sub-clinical or mildly diseased animals with no mechanical evidence of lung dysfunction. Although they are not feasible for routine clinical evaluation, they are valuable research tools.

Cytological evaluation of airway secretions collected by tracheal aspirate or BAL has become a mainstay in the diagnosis of chronic respiratory disease (Mair, 1987a; McGorum and Dixon, 1994; Moore and Cox, 1996).

Tracheal secretions are easily assessed and collected during bronchoscopy. Although tracheal aspiration is less invasive than BAL, tracheal secretion cytology does not correlate well with simultaneously collected BALF cytology (Derksen *et al.*, 1989) or histopathology in chronic lung disease (Larson and Busch, 1985). In a study of 300 cases of equine pulmonary disease (Dixon *et al.*, 1995c), despite there being a significant correlation between tracheal secretion and BALF neutrophil ratios in all disease categories, including COPD, substantial overlap in tracheal secretion neutrophil ratios was apparent between clinically normal and diseased horses. Moreover examination of BALF cytology permits much clearer definition of individual cells than in tracheal secretions which often contain large amounts of mucus. The molecular and cellular composition of the PELF that is collected by BAL appears to be homogeneous throughout different regions of the lung in both normal and COPD-affected horses (McGorum *et al.*, 1993e); hence a single BAL should be representative of the state of health of the entire lung. The collection, handling and interpretation of BALF is discussed in detail in Sections 2.7 and 2.8 and Chapter 6.

Several previous studies in both horses and humans have calculated PELF concentrations of cells and molecular components by assessment of the dilution factor in BALF based on endogenous references markers such as albumin or urea (Reynolds, 1987; McGorum *et al.*, 1993f). However, both of these techniques have the potential for significant error and many workers in the human field are moving away from methods to assess the proportion of PELF in BALF, suggesting quotation of the concentrations of BALF constituents simply on a per ml basis of recovered BALF (Klech and Pohl, 1989; Rennard *et al.*, 1998). The latter approach is used throughout the current study.

1.2 THE NEUTROPHIL

Although inter-species differences exist with respect to specific aspects of neutrophil structure and function (e.g. granule components, enzyme specificities, receptor expression, ligand recognition and eicosanoid synthesis), the universal role and capabilities of the polymorphonuclear granulocyte or neutrophil in the front line of host defence is common to all mammalian species. Until the study of equine neutrophil function has undergone the level of scrutiny enjoyed by its human counterpart, any discussion of equine neutrophil biology must rely heavily on comparative data. In this review, comparative data from human and experimental animal studies will be discussed as a model for the equine neutrophil, except where equine investigators have specifically addressed topics of discussion that are directly pertinent to the current study.

Neutrophils are characterized by their multilobulated nucleus and, like other granulocytes (eosinophils and basophils), by their distinctive cytosolic granules. Neutrophils constitute approximately 50% of the circulating leucocyte pool from whence they perform their primary task of seeking out, phagocytosing and destroying pathogens invading host tissues with rapidity and precision. Individuals in whom neutrophil function is deficient highlight the significance of efficient neutrophil function for host survival. Deficiency in neutrophil number

(neutropaenia), adhesion (leucocyte adhesion deficiency of cattle, dogs and humans), respiratory burst activity (chronic granulomatous disease, myeloperoxidase deficiency) or granule function (Chediak-Higashi syndrome) can lead to development of recurrent life-threatening bacterial and fungal infections (Gordon, 1994; Smith, 1994; Gerardi, 1996). However, the inappropriate or overexuberant release of the neutrophil's cytotoxic arsenal can also injure host tissues and is now implicated in the pathogenesis of many inflammatory diseases (Henson and Johnston, 1987; Smith, 1994; Dallegri and Ottonello, 1997. See Section 1.6).

1.2.1 ORIGIN, DEVELOPMENT AND CIRCULATION

All blood cells originate from pluripotent stem cells in the bone marrow. Neutrophils are derived from a common granulocyte-monocyte lineage, proliferating in about 5 cell divisions from this myeloblast stage through promyelocyte, myelocyte, metamyelocyte and band forms to become segmented neutrophils. Development is closely regulated by the cytokines GM-CSF, granulocyte-colony stimulating factor (G-CSF) and IL-3 (Tschudi *et al.*, 1975; Bainton, 1988; Gordon, 1994). Stages beyond the myelocyte are no longer capable of further differentiation and constitute the storage pool of the bone marrow from whence segmented neutrophils are released into the circulation. Characteristic azurophil granules form during the promyelocyte stage (Tschudi *et al.*, 1975) and their number per cell is reduced during subsequent mitoses (Bainton, 1988). In the healthy adult horse approximately 500 billion new granulocytes enter the circulation daily and have a half-life of 10.5 h (Carakostas *et al.*, 1981; Bainton, 1988). During an inflammatory response, transit time through the proliferating/mitotic phase and storage pool is decreased (Terashima *et al.*, 1996), the number of mitoses may be reduced (Sibille and Reynolds, 1990) and the subsequent circulating half-life increases (Carakostas *et al.*, 1981).

The total body pool of mature neutrophils is classically considered to be divided amongst the bone marrow storage pool, a circulating pool (accessible by venepuncture) and a marginated pool of temporarily sequestered cells which are in dynamic equilibrium with the circulating pool. Neutrophil margination is a

physiological phenomenon related to the lower pressures and pulsatile flow of the pulmonary circulation and the disparity in size between neutrophils and the capillaries through which they are passing. Depending on the species studied, 40-60% of pulmonary capillaries are narrower than the neutrophils passing through them (Doerschuk *et al.*, 1993). Consequently neutrophil transit is delayed as the cells deform in order to squeeze through, before re-entering the circulating pool. Neutrophils are also transiently retained in the capillaries of the bone marrow, liver and especially the spleen (Lien *et al.*, 1987; Hogg and Doerschuk, 1995; Peters, 1998).

The eventual fate of neutrophils reaching the end of their lifespan in healthy individuals is unclear, but recent *in vivo* cell labelling studies suggest that many are permanently retained within the spleen (possibly the lungs also) where they undergo apoptosis (Bicknell *et al.*, 1994).

1.2.2 STRUCTURE AND COMPONENTS

Normal equine neutrophils have a transectional area of approximately 100 μm^2 with an estimated diameter of 6-7 μm (Bertram and Coignoul, 1982). Light microscopic, ultrastructural and biochemical studies of neutrophils have demonstrated typical nuclear morphology, a small central Golgi complex, mitochondria, endoplasmic reticulum and characteristic populations of peroxidase-positive azurophilic granules, peroxidase-negative specific granules, gelatinase or tertiary granules and secretory vesicles (Bertram and Coignoul, 1982; Borregaard and Cowland, 1997). As well as proteolytic and microbicidal proteins, these storage organelles also contain a large number of preformed receptors and other membrane proteins, which, when incorporated into the plasma membrane following organelle fusion and exocytosis, can significantly alter neutrophil function (Borregaard and Cowland, 1997). A summary of pertinent granule and secretory vesicle contents is presented in Table 1.

Granule	Significant contents	Function of contents
Azurophil (primary)	Elastase	}
	Proteinase-3	} Serine proteinases
	Cathepsins	}
	Myeloperoxidase	Microbicidal
	Lysozyme	Hydrolase
	β-Glucuronidase	Hydrolase
	Defensins	Microbicidal
	Cationic proteins	Microbicidal
	α1-proteinase inhibitor	Elastase inhibitor
Specific (secondary)	CD11b/CD18b	Endothelial adhesion
	TNFReceptor	Priming and activation
	fMLP receptors	Chemotaxis
	G-protein sub-units	Signal transduction
	Cytochrome b_{558}	Respiratory burst oxidase
	Collagenase	} Metallo-
	Gelatinase	} proteinases
Gelatinase (tertiary)	CD11b/CD18	Adhesion
	Cytochrome b_{558}	Respiratory burst oxidase
	fMLP receptors	Chemotaxis
	Gelatinase	Metalloproteinase
Secretory vesicle	CD11b/CD18	Adhesion
	CD16 (FcγRIII)	Immunoglobulin recognition
	CD14	LPS recognition receptor
	Complement receptors 1 & 3	Complement recognition
	fMLP receptors	Chemotaxis
	Cytochrome b_{558}	Respiratory burst oxidase

Table 1.1: Contents of human neutrophil granules and secretory vesicles

This list of granule contents is not exhaustive; included are those with either direct relevance to the current study and/or those which have been studied in the horse (summarised in text). After Borregaard and Cowland (1997)

Ultrastructural examination has identified four types of equine neutrophil granules; azurophil, specific and two other distinct types (Bertram and Coignoul, 1982). The enzymatic contents of these cytosolic granules have received most attention, mainly in the search for *in vivo* markers of equine neutrophil degranulation.

Bochsler *et al.* (1992) identified release of lysozyme, β-glucuronidase, alkaline phosphatase, acid phosphatase and proteolytic and collagenolytic activity from equine neutrophils stimulated with phorbol ester *in vitro*. The granule fraction from homogenates of purified equine neutrophils were shown to contain 5'nucleotidase, alkaline phosphatase, acid phosphatase, α-mannosidase, β-glucuronidase, β-galactosidase and cytochrome c oxidase activity (Heyneman and Vercauteren, 1982). MMP-9 monomer and dimer levels were significantly elevated in synovial fluid collected from horses with septic arthritis and levels were correlated with the synovial fluid white blood cell count (Clegg *et al.*, 1997b). These same workers subsequently showed that neutrophils were a major source of MMP-9 (gelatinase), storing the enzyme intracellularly (Clegg *et al.*, 1997a).

Equine neutrophil myeloperoxidase (MPO) and serine proteinases have been extensively investigated. Two serine proteinases, equine neutrophil elastase (ENE) 2A and 2B, have been purified from neutrophils (Scudamore *et al.*, 1993; Pemberton *et al.*, 1993; Dubin *et al.*, 1994). Equine NE 2A has similar elastolytic activity to human neutrophil elastase, whereas that of ENE 2B is several fold higher (Potempa *et al.*, 1986). Equine NE 2A and 2B show 68% and 86% sequence homology with human neutrophil elastase, respectively (Dubin *et al.*, 1994). Early work suggested that equine neutrophils contained substantially less elastase than human neutrophils but recent work in this laboratory using a immunological technique indicates that equine neutrophils (0.8 pg/cell) actually contain similar amounts to their human counterparts (Dagleish *et al.*, 1999). Two intracellular ENE inhibitors, horse leucocyte elastase inhibitor and equine α-1-antiproteinase inhibitor (α-1PI), have also been described in the cytosol of equine neutrophils (Potempa *et al.*, 1991; Dagleish *et al.*, 1999).

Myeloperoxidase purified from equine neutrophils was shown to have similar electrophoretic and biochemical properties to human and bovine MPO (Mathy-Hartert *et al.*, 1998). The same workers subsequently developed a radioimmunoasay

for equine MPO and reported elevated levels of MPO in the plasma of horses with strangulating intestinal obstructions in comparison to animals with non-strangulating intestinal obstructions and normal horses (DebyDupont *et al.*, 1998; Grulke *et al.*, 1999).

1.3 ROLE OF NEUTROPHILS IN THE INFLAMMATORY RESPONSE

In order to perform their primary role of protecting the host from invading "foreign" cells, neutrophils must accumulate at sites of tissue injury and pathogen entry. This necessitates their extravasation from blood vessels by a co-ordinated series of events; initial margination within a vessel with temporary tethering and rolling of neutrophils along the endothelium prior to their arrest and firm adhesion, subsequent transmigration across the endothelium and through extracellular matrix delivers neutrophils to the inflammatory focus to phagocytose and kill micro-organisms.

1.3.1 NEUTROPHIL EXTRAVASATION

The mechanisms of neutrophil accumulation in the lung differ from those in the systemic circulation. Neutrophils invariably leave the systemic circulation via post-capillary venules whereas transit to the airspaces is achieved almost exclusively via the capillary system of the pulmonary circulation (Downey *et al.*, 1993; Rossi and Hellewell, 1994; Hogg and Doerschuk, 1995). The mechanisms of neutrophil migration from systemic vasculature which have been derived from *in vitro*, intravital microscopy and *in vivo* cell adhesion molecule monoclonal antibody blocking studies will be reviewed followed by a discussion of the contrasting features of transmigration in the lung.

1.3.1.1 Neutrophil margination and rolling

The release of components of invading micro-organisms (e.g. *N*-formyl-methionyl-leucyl-phenylalanine [fMLP] and LPS), secretion of locally generated inflammatory mediators (such as TNF-α, IL-8, PAF, LTB_4 and IL-1) or activation of complement

stimulate the adhesive capacity of endothelial cells and prime (q.v.) neutrophils, modulating their expression of adhesion molecules. Due to vasodilatation, flow in the blood vessel slows such that neutrophils move from the axial stream of blood cells to a more marginal course adjacent to vascular endothelium; also termed margination. Transient, loose adhesion events between neutrophils and endothelial cells cause the cells to "roll" along the vessel wall. These events are mediated by low-affinity interactions between the constitutively expressed L-selectin (CD62L) on neutrophil microvilli and sialyl Lewis X antigen (Sle^x, CD15) on endothelial cells. *De novo* expression of E-selectin and upregulation of P-selectin on the activated endothelial cell also contribute to this process. The passage of neutrophils is thus slowed but not stopped. Neutrophil adhesion molecule expression is tightly regulated by both soluble and activated endothelial cell-associated cytokine levels such that L-selectin is shed as intracellular granules fuse with the neutrophil plasma membrane, increasing expression and avidity of the β_2-integrin CDl1b/CD18 (Carlos and Harlan, 1994; Rossi and Hellewell, 1994; Hogg and Doerschuk, 1995; Condliffe *et al.*, 1996).

1.3.1.2 Neutrophil arrest and firm adhesion

Binding of CDl1b/CD18 to its ligand, Inter Cellular Adhesion Molecule-1 (ICAM-1), newly upregulated on activated endothelial cells, leads to arrest of the neutrophil on the endothelial surface and firm adhesion. This stable binding is also supported by homophilic binding of another member of the immunoglobulin superfamily, platelet-endothelial cell adhesion molecule (PECAM)-1, expressed on both leucocytes and endothelium, and by bridging of acute phase molecules such as fibrinogen and complement fragments between CDl1b/CD18 and either ICAM-1 or directly to the endothelial cell surface (Carlos and Harlan, 1994). The apparent redundancy of mechanisms to facilitate firm adhesion of neutrophils emphasises its importance in the extravasation process. Although the above paradigm has yet to be confirmed in the horse, equine neutrophils express CDl1b/CD18 and adhere to both serum- and fibronectin-coated plastic in a CD18-dependent manner (Bochsler *et al.*, 1990; Marr *et al.*, 1999). Moreover, adherence is enhanced by chemokines (IL-8),

complement fragments (C5a) and lipid inflammatory mediators such as PAF and LTB_4 (Foster *et al.*, 1997; Marr *et al.*, 1999).

1.3.1.3 Neutrophil shape change, transendothelial migration and chemotaxis

The change from selectin- to integrin-dependent adhesion coincides with the normally spherical neutrophil becoming activated and motile, appearing ruffled, flattened and polarised. Microfilamentous (F-) actin that makes up much of the intracellular cytoskeleton is intimately associated with both the plasma membrane and many of its integral chemoattractant receptors and adhesion molecules, including integrins. Receptor engagement stimulates actin polymerisation with addition of actin monomers to the F-actin in a directed fashion toward the inciting stimulus, thereby deforming the plasma membrane to produce a leading broad lammelipodium and a thin trailing uropod. The polarized cell can then "crawl" along a chemotactic gradient by constant cycling of actin monomers from the tail of the actin filaments back to the point of membrane attachment and polymerization at the leading edge (Snyderman and Goetzl, 1981; Bearer, 1993; Rosales and Juliano, 1995). A chemotactic gradient is necessary for trans-endothelial migration to follow after adhesion to the endothelium. The polarized cell, guided by PECAM-1 localised at inter-endothelial cell junctions and aided by the high affinity binding between CDl1b/CD18 and ICAM-1, migrates either between or directly through endothelial cells to pause transiently before breaching the endothelial basement membrane (DeLisser and Albelda, 1998; Feng *et al.*, 1998).

Dissolution of basement membrane is achieved, at least in part, by the tightly focussed activity of neutrophil MMPs activated *in situ* by elastase either expressed on the neutrophil's surface or released locally (Owen *et al.*, 1995; Delclaux *et al.*, 1996; Kumagai *et al.*, 1999).

Finally neutrophils migrate through the extravascular interstitial matrix along a chemotactic gradient toward the inflammatory stimulus. This is achieved by a chain of repetitive engagement and disengagement between extracellular matrix glycoproteins such as fibrinogen and fibronectin and both β1- (CD11a/CD18) and β_2-integrins that integrate with the cytoskeleton. Granule exocytosis is preferentially focussed on the leading edge of the lamellipodium, providing the ability for directed

movement along a chemotactic gradient (Clapp *et al.*, 1994; Rosales and Juliano, 1995; Werr *et al.*, 1998). Indeed, repetitive engagement and cross-linking of β_2-integrins may act as a further priming stimulus prior to initiation of phagocytosis and the respiratory burst (Liles *et al.*, 1995).

1.3.1.4 Neutrophil extravasation in the lung

The leucocyte-endothelial cell adhesion cascade described above is now generally accepted to underpin neutrophil extravasation from post-capillary venules in the systemic vascular beds. However, the use of blocking antibodies, antisense oligonucleotides and mutant mice with targeted deletion of specific adhesion molecules has demonstrated that in some circumstances neutrophil emigration from the pulmonary circulation into the interstitium or alveolar space occurs without involvement of selectins, β_2-integrins or ICAM-1 (Wegner, 1994; Hogg and Doerschuk, 1995; DeLisser and Albelda, 1998). The requirement for β_2-integrins in particular, appears to be governed by the inflammatory stimulus employed. For example, pulmonary neutrophil accumulation in response to *Escherichia coli* and LPS is β_2-integrin-dependent whereas the responses to *Streptococcus pneumoniae* and C5a are not (Doerschuk *et al.*, 1990). As discussed in Section 1.2.1, the disparity in diameter between neutrophils and pulmonary capillaries underlies the physiological sequestration of the marginated pool of neutrophils in the lung. Sequestration is also governed by biomechanical properties of the neutrophil, particularly cell size and stiffness or deformability (Downey *et al.*, 1990; Selby *et al.*, 1991). During an inflammatory response, stimulation of neutrophils by local or circulating inflammatory mediators causes an increase in their cell volume and a reduction in deformability, thereby slowing neutrophil transit time through pulmonary capillaries still further. These features alone may facilitate arrest of stimulated neutrophils in direct contact with activated endothelial cells for sufficient time to initiate the process of transendothelial migration outlined in Section 1.3.1.3 (Worthen *et al.*, 1989; Downey *et al.*, 1993; Hogg and Doerschuk, 1995; DeLisser and Albelda, 1998).

1.3.2 NEUTROPHIL PHAGOCYTOSIS AND THE RESPIRATORY BURST

Having migrated into tissues, the neutrophil utilises a series of mechanisms for the recognition, engulfment and intracellular destruction of foreign material. The engulfment process (phagocytosis) and/or exposure to locally generated secretagogue stimuli lead to assembly of the respiratory burst oxidase at either the cell and/or phagolysosomal (surrounding ingested material) plasma membrane. Neutrophil activation or "triggering" of the respiratory burst oxidase initiates a massive increase in cellular oxygen consumption (hence "respiratory burst") and the elaboration of microbicidal reactive oxygen species (ROS). In this thesis, the use of the term "activation", as it pertains to neutrophils, will be restricted to activation of the respiratory burst.

1.3.2.1 Phagocytosis

Phagocytes, including neutrophils, recognize and phagocytose large particles (>0.5 μm) by one of three basic mechanisms. Complement receptor 3 (CR3, CD11b/CD18) on phagocytes can recognize some pathogenic agents (e.g. bacteria such as *E. coli*, *streptococci* and *staphylococci* and bacterial fragments such as LPS) by direct interaction with LPS and bacterial surface glycoproteins. However, recognition and phagocytosis is achieved more usually and indeed much more efficiently, if particles have been previously opsonised by complement or specific immunoglobulin. Particles opsonised with immunoglobulin are bound by Fcγ receptors (FcγR's I, II and III) whereas those opsonised with complement (C3bi) bind to CR3. It is important to note that prior neutrophil stimulation/priming will have already increased the expression of these as well as many other surface receptors through exocytosis of specific granules and secretory vesicles. Although the mechanisms of particle uptake vary slightly (membrane ruffling and pseudopod extrusion initiate FcγR-mediated uptake, whereas complement-coated prey sink into the cell surface after receptor engagement), the re-organisation of cytoskeletal F-actin facilitates enclosure of the ingested particle within a phagolysosome (Hellewell and Williams, 1994; Caron and Hall, 1998).

1.3.2.2 Activation of the respiratory burst

Although it is clear that activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (respiratory burst oxidase) following phagocytosis underpins the microbicidal capacity of the neutrophil; the precise methods by which micro-organisms are killed is not clear (Babior *et al.*, 1973; Weiss, 1989; Rosen *et al.*, 1995; Wientjes and Segal, 1995).

The NADPH oxidase catalyses the generation of superoxide anions (O_2^-, the precursor for the neutrophil's arsenal of ROS) by the one-electron reduction of oxygen in which NADPH acts as the electron donor.

$$2O_2 + NADPH \xrightarrow[\text{Oxidase}]{\text{NADPH}} 2O_2^- + NADP^+ + H^+$$

The activated enzyme is composed of five subunits: $p40^{PHOX}$, (PHOX = PHagocyte OXidase), $p47^{PHOX}$, $p67^{PHOX}$, $p22^{PHOX}$ and $gp91^{PHOX}$. When cells are quiescent, the enzyme is maintained in the inactive state by physical separation of its components; $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$ forming a complex in the cytosol whilst $p22^{PHOX}$ and $gp91^{PHOX}$ occur as a heterodimeric flavohaemoprotein (Cytochrome b_{558}) in the membranes of specific granules and secretory vesicles. Neutrophil stimulation leads to phosphorylation of $p47^{PHOX}$ followed by migration of its entire complex to the cell's plasma membrane or plasma membrane that has been incorporated into a phagolysosome. Cytochrome b_{558} is delivered to the plasma membrane by fusion and exocytosis of specific granules and secretory vesicles where, co-ordinated by two low molecular weight guanine nucleotide-binding proteins (Rac2 and Rap1A), the complete oxidase is assembled and superoxide anions are discharged into the extracellular milieu or the phagolysosome (Weiss, 1989; Wientjes and Segal, 1995; Babior, 1999).

The free radical superoxide anion may dismutate, either spontaneously or be catalysed by intracellular superoxide dismutase:

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + 0_2$$

Superoxide anions and hydrogen peroxide may themselves mediate microbial killing but it seems likely that further oxidation generates more toxic ROS (Weiss, 1989; McColl and Showell, 1994; Rosen *et al.*, 1995). In the neutrophil, MPO can oxidize halide ions (typically chloride) in the presence of hydrogen peroxide to form highly toxic hypohalous acids (Weiss, 1989; Anderson *et al.*, 1997; Ochoa *et al.*, 1997):

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCl + H_2O$$

Although great contention has surrounded the neutrophil's ability to generate the highly reactive hydroxyl radical (HO•), this does appear to occur by interaction of superoxide anions with redox sensitive metal ions (probably iron molecules supplied by lactoferrin) via the Haber-Weiss reaction (Weiss, 1989; Rosen *et al.*, 1995):

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \longrightarrow HO\bullet + HO^- + Fe^{3+}$$

Once this cascade of ROS generation begins, many of the products generated, as well as being directly microbicidal, can also react with other intracellular molecules to produce further toxic products. For example, superoxide anions can react with nitric oxide to produce peroxynitrite and the H_2O_2-MPO-chloride system can convert the hydroxy-amino acids threonine and serine to the extremely reactive aldehydes, glycoaldehyde, 2-hydroxypropanal and acrolein (Rosen *et al.*, 1995; Anderson *et al.*, 1997).

Despite the elaboration of an impressive range of oxidative cell toxins, auto-oxidative stress-induced cell injury is prevented by both the compartmentalisation of these reactions within phagolysosomes and by the presence of intracellular anti-oxidants such as ascorbate and glutathione. However, in certain circumstances, the cell's complement of ROS may be turned to teleological advantage by initiating the programme of apoptosis following ingestion of potentially pathogenic particles (Coxon *et al.*, 1996; Watson *et al.*, 1996b).

1.3.2.3 Degranulation

It is clear from the preceding review of neutrophil extravasation and respiratory burst activity that degranulation or exocytosis of intracellular granule contents is critical for the appropriate interaction of the neutrophil with its surroundings. Granule contents facilitate recognition, interaction and reaction with extracellular stimuli. Moreover, degranulation responses are graded to reflect the nature and magnitude of the inciting stimulus. Granules are recruited to the plasma membrane in a hierarchical fashion. This is governed principally by a combination of the sensitivity of granules to intracellular signalling transients such as increases in intracellular calcium and the density of plasma membrane attachment or fusion proteins in the granule membrane (Borregaard and Cowland, 1997). Thus secretory vesicles, delivering adhesion molecules and chemoattractant receptors to the plasma membrane are mobilised most readily, followed by gelatinase granules. Despite an apparent cascade of granule exocytosis to match the cell's functional requirements during its path from vascular sequestration to activation in tissues, the regulation of granule recruitment is capable of exquisite fine tuning. The exocytosis of neutrophil elastase from azurophil granules provides a good example. Priming agents can increase the surface expression of elastase molecules without release into the extracellular milieu (Owen *et al.*, 1997). Whereas secretion of elastase can vary from exocytosis of the contents of a single azurophil granule to release of much of the cell's protease load (Liou and Campbell, 1996; Owen and Campbell, 1999).

1.3.3 REGULATION AND AMPLIFICATION OF THE INFLAMMATORY RESPONSE

Although neutrophils are key effector cells, they are also capable of a significant regulatory role both locally at the site of inflammation and at distant sites through the release of soluble mediators. The terminally differentiated neutrophil retains considerable capacity for *de novo* cytokine synthesis and release (Cassatella, 1995) and neutrophil respiratory burst products may themselves act as local cell signalling molecules or generate further chemotactic agents by cleavage of local substrates (Henson and Johnston, 1987).

In vitro, stimulation of neutrophils by a variety of agents can induce synthesis and release of further pro-inflammatory stimuli such as TNF-α, IL-1β, IL-8, IL-6, PAF and LTB_4, thereby amplifying the inflammatory response and potentially recruiting a further wave of neutrophils to the inflamed site (Bazzoni *et al.*, 1991; Baggiolini *et al.*, 1994; Furie and Randolph, 1995). Models of LPS-induced acute lung inflammation in rodents demonstrate the significance of neutrophil-derived cytokines in the amplification of inflammation *in vivo*. The early phase (<6h) of the inflammatory response is associated with cytokine expression in alveolar macrophages. However, at later time points (6-12 h) neutrophils become the dominant source of TNF-α, IL-1β, IL-6 and MIP-2 (Xing *et al.*, 1993; Xing *et al.*, 1994; Wohlford-Lenane *et al.*, 1999). Moreover, cytokine synthesis appeared to be a feature specific to airspace neutrophils and was not recognized in neutrophils sequestered in the pulmonary vasculature. Neutrophils also constitutively expressed transforming growth factor-β (TGF-β), a cytokine intimately involved in tissue repair.

Neutrophil-derived ROS may also contribute to further granulocyte recruitment. Reaction of superoxide anions with nitric oxide forms peroxynitrite, a potent neutrophil priming agent (Rohn *et al.*, 1999). Also neutrophil-derived oxidants stimulate generation, enhanced surface expression and release of PAF from endothelial cells as well as increasing expression of P-selectin with the potential to enhance neutrophil recruitment and extravasation (McIntyre *et al.*, 1995).

1.4 NEUTROPHIL PRIMING

Regulation of neutrophil function to prevent premature or inappropriate activation of the respiratory burst whilst facilitating neutrophil accumulation at sites of inflammation is paramount to minimise inadvertent host tissue injury. A key regulatory feature of neutrophil function is the concept of neutrophil "priming" as an intermediate step in the progression from a circulating quiescent cell to triggering of the respiratory burst in a fully activated cell recruited to an inflammatory focus (Lehrer *et al.*, 1988; Smith, 1994). Priming, although initially defined as an *in vitro*

phenomenon (Guthrie *et al.*, 1984), is now recognized as a prerequisite for efficient neutrophil recruitment and paradoxically, in the pathogenesis of neutrophil-mediated host tissue injury (Smedly *et al.*, 1986; Worthen *et al.*, 1987; Williams *et al.*, 1993). "Priming" is defined as a mechanism whereby the response of the neutrophil to a subsequent ("activating") stimulus is synergistically enhanced following exposure to a prior (priming) stimulus (Downey *et al.*, 1995; Condliffe *et al.*, 1998b).

Both physiological pro-inflammatory mediators such as bacterial products, cytokines and lipid mediators and physico-chemical changes in the surrounding environment may prime neutrophils. The latter are important considerations in the purification of neutrophils for *in vitro* studies, wherein excessive cell trauma during venepuncture and subsequent manipulation or exposure to markedly hypotonic conditions (Edashige *et al.*, 1993; Kitchen *et al.*, 1996b) can prime cells.

In vitro, physiological priming stimuli may initiate priming only ("dedicated primers", e.g. PAF, TNF-α) or may prime cells at low concentrations but directly activate the respiratory burst at higher concentrations (e.g. C5a). Both the response to an individual priming agent and the time required to initiate the primed state can vary greatly. This may, at least in part, reflect engagement of different receptors and intracellular signalling pathways. The majority of physiological priming agents signal through one of three main plasma membrane receptor types. 1. The classical chemoattractant, seven transmembrane spanning domain, heterotrimeric G-protein-linked, receptors (e.g. PAF, fMLP, C5a, IL-8). 2. Integral single transmembrane domain cytokine/growth factor receptors linked to the cell interior by tyrosine phosphorylation pathways (e.g. TNF-α, GM-CSF). 3. Adhesion molecules that require spatial clustering, cross-linking or ligation to initiate signalling (e.g. β_2-integrins and selectins) (Bokoch, 1995; Downey *et al.*, 1995; Liles *et al.*,1995).

A review of the biological properties of the putative equine neutrophil priming agents investigated in the current study is presented in Chapter 3.

The intracellular signalling pathways and second messengers contributing to the mechanisms of neutrophil priming and activation remain under intense debate and are outwith the scope of the current work, but have been recently reviewed (Pabst, 1994; Bokoch, 1995; Downey *et al.*, 1995; Condliffe *et al.*, 1998a; Condliffe *et al.*, 1998b).

1.4.1 CONSEQUENCES AND INDICES OF NEUTROPHIL PRIMING

The initial *in vitro* description of neutrophil priming by Guthrie and colleagues (1984) demonstrated significantly enhanced (10-20 fold) neutrophil oxidant generation (superoxide anions and H_2O_2) in response to immune complexes, phorbol 12-myristate 13-acetate (PMA) and fMLP following pre-incubation with LPS. It is thought that circulating quiescent human neutrophils are entirely unresponsive to fMLP (Pabst, 1994) and neutrophils isolated under optimal conditions are minimally responsive to this formyl peptide (Haslett *et al.*, 1985). Consequently, the effect of priming agents on agonist-induced (particularly fMLP) superoxide anion generation has become the gold standard for the investigation of neutrophil priming both *in vitro* and *ex vivo* (Haslett *et al.*, 1985; Kitchen *et al.*, 1996b; Condliffe *et al.*, 1998b). Review of the mechanisms of neutrophil extravasation (Section 1.3.1) clearly suggests that the functional changes required to initiate neutrophil margination and adhesion are likely to be intimately associated with priming.

Exposure of neutrophils to inflammatory mediators, including priming agents, *in vitro* stimulates cell shape change (Haslett *et al.*, 1985; Kitchen *et al.*, 1996a) and decreases their deformability (Worthen *et al.*, 1989). Not only is the extent of neutrophil shape change predictive for their subsequent chemotactic capacity (Haston and Shields, 1985) and fMLP-stimulated superoxide anion generation (Haslett *et al.*, 1985; Kitchen *et al.*, 1996b) but the associated cell stiffening (lower deformability) is also a critical determinant of neutrophil retention in pulmonary capillaries *in vivo* (Worthen *et al.*, 1989; Selby *et al.*, 1991; Ussov *et al.*, 1996).

The ability of priming agents to regulate human neutrophil adhesion molecule expression and to enhance agonist-induced adhesion *in vitro* is well recognized (Haslett *et al.*, 1985; Young *et al.*, 1990; Condliffe *et al.*, 1996) and modulation of equine neutrophil adhesion molecule expression and function by such agents has been described (Bochsler *et al.*, 1990; Foster *et al.*, 1997; Marr *et al.*, 1999). Not only does priming regulate neutrophil adhesion *per se* (Condliffe *et al.*, 1996) but the bi-directional interaction between neutrophil and endothelial cell integrins, selectins and their ligands can also act as neutrophil priming stimuli (Liles *et al.*, 1995; Ruchaud-Sparagano *et al.*, 1998). Therefore priming of intravascular neutrophils

may facilitate neutrophil margination and arrest in the microvasculature with subsequent adhesion molecule-dependent neutrophil transmigration providing a further priming stimulus to enhance respiratory burst activity on arrival at the inflammatory focus.

Agonist-induced lipid mediator release (e.g. LTB_4, arachidonic acid) can also be augmented (Doerfler *et al.*, 1994), with the potential to enhance subsequent neutrophil recruitment/chemotaxis (LTB_4) and activation (arachidonic acid).

Priming agents appear to have dual effects on the regulation of neutrophil-mediated proteolytic activity. Firstly, and perhaps of prime pathogenetic importance, is the ability of priming agents to greatly enhance agonist-stimulated membrane-bound elastase expression (Owen *et al.*, 1997). Retention of membrane-bound serine proteinases appears to be a critical mechanism for the maintenance of focussed proteolytic activity in the presence of protease inhibitors and may be singularly important in both transendothelial cell migration and host tissue injury (Owen *et al.*, 1995; Owen and Campbell, 1999). Degranulation with extracellular secretion of granule enzymes is an integral part of neutrophil activation and ROS generation (Section 1.3.2.3). However, initial investigation of LPS priming of agonist-stimulated azurophil granule exocytosis in both human and equine neutrophils suggested that although secretion was enhanced by priming, these responses were substantially less "primable" than superoxide anion generation (Haslett *et al.*, 1985; Fittschen *et al.*, 1988; Bochsler *et al.*, 1992). This discrepancy remained unexplained until recently when pre-incubation with LPS in the presence of 1% serum (in contrast to the previous studies) was reported to enhance fMLP-stimulated elastase release from human neutrophils nearly 20-fold (Ottonello *et al.*, 1997). We have recently produced similar data with equine neutrophils (T.J. Brazil and M.P. Dagleish, unpublished observations). These findings illustrated two points. Firstly, the importance of serum components in the recognition mechanism of LPS by neutrophils (reviewed in Chapter 3) and secondly the significance of priming for neutrophils to achieve their full microbicidal potential.

1.4.2 NEUTROPHIL PRIMING *IN VIVO*

Neutrophil priming has been reported in a plethora of clinical inflammatory disease states and experimental animal models.

Most heartening for the laboratory investigator is confirmation that detection of primed peripheral blood neutrophils *in vivo* is correlated with the pathophysiological consequences predicted by *in vitro* priming models. A clear example of this is the strong correlation between the proportion of shape changed neutrophils in peripheral blood (assessed *ex vivo*) and the size of the sequestered pulmonary granulocyte pool in extrapulmonary inflammatory diseases (Ussov *et al.*, 1996).

In vitro modelling receives further credence from *in vivo* studies demonstrating priming of peripheral blood neutrophils following experimental infusion of well documented *in vitro* neutrophil priming agents. Intravenous administration of LPS (Cerasoli *et al.*, 1990) and TNF-α (Wewers *et al.*, 1990; Kapp *et al.*, 1991) in human subjects and LPS in horses (Krumrych *et al.*, 1996) enhances peripheral blood neutrophil respiratory burst activity. Of note, enhanced neutrophil oxidative activity was also induced by inhalation of LPS by human subjects (Michel *et al.*, 1995).

Primed neutrophils have been reported in the blood of human patients with vasculitis and inflammatory bowel disease (Ussov *et al.*, 1996), bacterial infections (Bass *et al.*, 1986; Follin *et al.*, 1989), acute respiratory distress syndrome (ARDS) (Zimmerman *et al.*, 1983; Chollet-Martin *et al.*, 1992) and allergic asthma (Meltzer *et al.*, 1989). Indeed, in human ARDS, the degree of peripheral blood neutrophil priming (fold enhancement of H_2O_2 release compared to control patients) correlated with the severity of lung injury (Chollet-Martin *et al.*, 1992). Severe trauma rapidly (3-6 h) primes neutrophils in human patients and this may be a major risk factor for their subsequent development of multiple organ failure and ARDS (Botha *et al.*, 1995).

Elevated circulating levels of potential neutrophil priming agents (e.g. TNF-α, IL-8, LPS) correlated with both the degree of neutrophil priming and the severity of lung injury in human ARDS (Chollet-Martin *et al.*, 1992; Pittet *et al.*, 1997), are an important prognostic indicator for the development of ARDS in high risk human patients (Donnelly *et al.*, 1993) and were correlated strongly with mortality in horses with acute inflammatory or ischaemic intestinal diseases (Steverink *et al.*, 1995).

Functional assessment of both circulating and extravasated neutrophils is a critically important aspect of dissecting the pathogenesis of neutrophil mediated diseases (Pittet *et al.*, 1997). However, it is important to note that although priming significantly enhances pulmonary sequestration of neutrophils, this does not necessarily lead to neutrophil extravasation or indeed lung injury (Martin *et al.*, 1989; Garat *et al.*, 1995; Ussov *et al.*, 1996).

1.5 APOPTOSIS AND CLEARANCE OF EFFETE NEUTROPHILS

Although much study has focussed on the mechanisms underlying the kinetics (Boggs, 1967), recruitment and accumulation (Arai *et al.*, 1990; Carlos and Harlan, 1994; Rossi and Hellewell, 1994) of neutrophils during an inflammatory response, the eventual fate and disposal of the amassed load of neutrophils at the inflamed site has only been addressed relatively recently (Haslett, 1992). Physical clearance mechanisms may be important in certain body systems, such as expulsion of cells and debris from the respiratory tract by the mucociliary apparatus and cough reflex and emigration of neutrophils via the circulation in the renal vasculature (Hughes *et al.*, 1997). For many years it was assumed that the majority of recruited neutrophils met their fate in inflamed tissues by undergoing necrosis and disintegration, with their fragments being scavenged by macrophages (Hurley, 1983). However, such a fate would inevitably lead to local host tissue damage following liberation of the cells' injurious contents.

The observations of Newman and co-workers (1982) however, indicated that as neutrophils "aged" in culture they came to be recognized, ingested intact and rapidly degraded by macrophages, quite unlike freshly isolated cells. This led to the discovery that the critical permissive step in this clearance mechanism was in fact the capacity of the neutrophil to undergo constitutive apoptosis or programmed cell death (Savill *et al.*, 1989b). Identification of the stereotypical features of nuclear chromatin condensation and pyknosis (Wyllie *et al.*, 1980b) and strictly ordered internucleosomal DNA cleavage (Wyllie, 1980a; Hale *et al.*, 1996) confirmed the apoptotic phenotype (reviewed further in Chapter 4). This has led to a major

research effort to understand the regulation of the age-dependent and constitutive programme of neutrophil apoptosis and subsequent macrophage recognition, with a view to developing new therapeutic strategies that could facilitate this beneficial process and thereby counter neutrophil-mediated tissue injury (Haslett, 1997; Savill, 1997a).

The modulation of the constitutive rate of neutrophil apoptosis by physiological inflammatory stimuli is reviewed and discussed in Chapter 4 in the light of data generated in the current study of equine neutrophils.

1.5.1 APOPTOSIS

Apoptosis was initially defined and differentiated from cell death leading to necrosis by its distinct morphological phenotype. Cellular swelling, disintegration of the nucleus and cytoplasmic organelles and loss of plasma membrane integrity, frequently associated with a surrounding inflammatory response, are common features of cell necrosis. In contrast, cells undergoing apoptosis shrink and membrane integrity is maintained as their nuclear chromatin condenses into one or more dense "pyknotic" globes prior to their non-phlogistic elimination by adjacent phagocytes (Kerr *et al.*, 1972; Wyllie *et al.*, 1980b; Kerr *et al.*, 1995; Majno and Joris, 1995). This formulaic, genetically programmed, form of cellular suicide drives many forms of "physiological" cell deletion. This ranges from tissue remodelling during embryological development, through cellular renewal of evanescent tissues (e.g. intestinal epithelia) to the elimination of cells marked by malignant transformation, immune selection or viral infection (Kerr *et al.*, 1972; Wyllie *et al.*, 1980b; Ben-Sasson *et al.*, 1995; Hale *et al.*, 1996). The ordered fragmentation of chromatin into 180-200 bp multiples by endonucleases that belies its morphological condensation and produces the characteristic ladder pattern on electrophoresis of DNA is recognized as the defining biochemical feature of apoptosis (Wyllie, 1980a; Bortner *et al.*, 1995; Squier *et al.*, 1995).

1.5.2 INTRACELLULAR MECHANISMS AND REGULATION OF APOPTOSIS

Apoptosis may be stimulated by cell injury (e.g. ionising radiation, viral infection, and elaboration of granzyme B by cytotoxic T lymphocytes) and physiological stimuli of either cell surface receptors and/or transcriptional mechanisms (Ashkenazi and Dixit, 1998) or it may be constitutive, as observed in granulocytes. However, two features of the mechanics of apoptosis are clear. Firstly, irrespective of the inciting stimulus, the final common pathway of cell death is consistent. Secondly, in contrast to necrotic cell death, cells undergoing apoptosis actively participate in a genetically predetermined suicide. In non-haematopoetic cells, ablation of protein or RNA synthesis delays or blocks apoptosis, confirming that it is an active process requiring translation of certain gene products (Wyllie *et al.*, 1984). In granulocytes however, the reverse is true; apoptosis is rapidly promoted by inhibition of protein synthesis (Whyte *et al.*, 1997), suggesting that granulocytes' inexorable progression to apoptosis is temporarily checked by synthesis of survival proteins.

Much of the current knowledge regarding the molecular regulation of apoptosis and the phagocytosis of dead cells has come from studies of the nematode *Caenorhabditis elegans* in which 131 of its 1090 adult somatic cells undergo apoptosis during development (Hale *et al.*, 1996). Targeted gene mutation has defined the genes specifically responsible for initiation of apoptosis, suppression of cell death, phagocytosis of dead cells and finally their digestion. Although the molecular basis of apoptosis in mammalian cells is more complex, with many more gene products involved in control of each of the phases described above, structural and functional mammalian homologues of some terminal effector molecules have now been defined. It is now clear that the structural changes observed in apoptosis are universally driven by a family of proteases with cysteine at their active site cleaving peptides at an aspartate residue, collectively termed "caspases" (Barinaga, 1998; Thornberry and Lazebnik, 1998). Furthermore, in most healthy living cells caspase activity and hence apoptosis is actively suppressed by a family of molecules whose key players are bcl-2 and bcl-x_L (Adams and Cory, 1998).

Intracellular signalling in apoptosis and the gene products regulating caspase family activity remain areas of intense study and are subject to regular review (Bellamy *et*

al., 1995; Eastman, 1995b; Hale *et al.*, 1996; Barinaga, 1998; Adams and Cory, 1998; Thornberry and Lazebnik, 1998; Ashkenazi and Dixit, 1998) but are beyond the scope of this review.

1.5.3 FUNCTIONAL CONSEQUENCES OF NEUTROPHIL APOPTOSIS

It appears that the pathway from commitment to apoptosis, to macrophage recognition has evolved a plethora of mechanisms to limit the pro-inflammatory capacity of effete neutrophils in inflamed tissue. These mechanisms help to maintain the fine balance between successful anti-microbial defence and collateral host tissue injury. A key feature is the maintenance of neutrophil plasma membrane integrity long after the initiation of the apoptotic programme, hence preventing leakage of noxious contents from the dying cell (Savill *et al.*, 1989b). Specific apoptosis-related changes in cell surface phenotype serve to limit neutrophil effector functions such as adhesion (Dransfield *et al.*, 1995) and secretory activity (Whyte *et al.*, 1993b) through down-regulation of both receptor expression and function. This effectively insulates the apoptotic neutrophil from pro-inflammatory stimuli in the surrounding milieu. Further discussion of the functional consequences of neutrophil apoptosis is presented in Chapter 4 in the context of functional data from apoptotic equine neutrophils.

1.5.4 MACROPHAGE RECOGNITION OF APOPTOTIC NEUTROPHILS

Expression of novel molecules on the cell's exterior, such as the membrane aminophospholipid, phosphatidylserine (Homburg *et al.*, 1995), appears to "flag up" the dying cell as being ready for macrophage tethering and subsequent elimination (Savill, 1998). Indeed, some alterations in cell surface phenotype are so profound and specific that they now underpin highly sensitive assays for the definition of apoptosis in individual neutrophils (Dransfield *et al.*, 1994; Martin *et al.*, 1995). Unlike phagocytosis of many other particles, the recognition mechanisms initiating ingestion of apoptotic neutrophils by macrophages do not stimulate secretion of pro-inflammatory mediators, such as arachidonic acid derivatives or cytokines (Meagher

et al., 1992; Fadok *et al.*, 1998). In fact, uptake of apoptotic cells, at least *in vitro*, appears to actively suppress the generation of pro-inflammatory cytokines via an autocrine/paracrine feedback loop (Fadok *et al.*, 1998). Furthermore, the very inflammatory mediators (for example GM-CSF and TNF-α) that enhance neutrophil function and regulate neutrophil longevity may act simultaneously to recruit more macrophages into a clearance role as well as increasing their "appetite" for apoptotic neutrophils (Ren and Savill, 1995).

The capacity of resident or inflammatory phagocytes to dispose of apoptotic neutrophils is considerable, with individual macrophages being capable of ingesting multiple effete cells via a number of discrete recognition mechanisms (Hart *et al.*, 1996). A further fail-safe disposal mechanism is offered by a number of other cell types, not usually phagocytic in nature, which may be recruited into a clearance role; the so-called "semi-professional" phagocytes, such as renal mesangial cells (Savill *et al.*, 1992). The recognition that several highly efficient recognition mechanisms are available to a single macrophage phenotype (Savill, 1997b) further suggests that macrophage clearance of apoptotic neutrophils represents a key damage-limiting feature in the successful resolution of an inflammatory focus.

The initial characterization of human neutrophil apoptosis *in vitro* by Savill and colleagues (1989) identified morphologic phenotypes in ageing neutrophils identical to those that had been recognized *in vivo* in inflamed lungs, joints and glomeruli. Due to the rapidity with which effete cells are recognized, engulfed and degraded by macrophages (probably < 60 min) (Duvall *et al.*, 1985; Bursch *et al.*, 1990; Majno and Joris, 1995), estimates of apoptosis rates *in vivo* derived from static observations (i.e. histological or cytological preparations) are likely to considerably underestimate its importance (Savill, 1997a).

Observations from rodent models of inflammation such as LPS- (Cox *et al.*, 1995) and oleic acid-induced lung injury (Hussain *et al.*, 1998), and from human inflammatory diseases such as the acute respiratory distress syndromes of neonates (Grigg *et al.*, 1991) and adults (Matutè-Bello *et al.*, 1997) have provided compelling evidence for apoptosis as a pivotal regulator of neutrophil-mediated inflammation. Moreover, pharmacological manipulation of apoptosis and macrophage clearance

already shows considerable promise in the therapy of eosinophil-mediated inflammation (Tsuyuki *et al.*, 1995; Woolley *et al.*, 1996).

1.6 ROLE OF NEUTROPHILS IN HOST TISSUE INJURY

The benefits to host defence afforded by the neutrophil's armoury of microbicidal secretory products are, paradoxically, frequently overshadowed by the detrimental effects of these indiscriminate cytotoxins on host tissues. This is particularly so in a plethora of non-septic inflammatory diseases of humans such as vasculitis, ARDS, rheumatoid arthritis, glomerulonephritis, psoriasis, inflammatory bowel disease, asthma and myocardial infarction in which neutrophil products have been implicated (Henson and Johnston, 1987; Malech and Gallin, 1987; Haslett, 1992; Smith, 1994). Furthermore, the pathogenetic significance of neutrophil-derived cytotoxins is now well recognized in equine medicine, particularly in intestinal reperfusion and mucosal injury (Laws and Freeman, 1995; Moore *et al.*, 1995; Inoue *et al.*, 1998) and septic arthritis (MacDonald and Benton, 1996; Clegg *et al.*, 1997a).

It is important to emphasise that such effects may be subtle and the significance of functional cell injury may be eclipsed in the search for structural pathological correlates of neutrophil activation (Henson and Johnston, 1987). For example, hypochlorous acid-induced increases in equine colonic mucosal permeability may have significant consequences for intestinal function in the absence of major structural pathology (MacDonald and Benton, 1996). Similarly, pulmonary gas exchange is likely to be particularly sensitive to compromise of alveolar epithelial cell function (Votion *et al.*, 1998).

Sections 1.2 and 1.3 reviewed the range of potentially injurious neutrophil products. Although many neutrophil-derived oxidants have been directly or indirectly implicated in mammalian cytotoxicity both *in vitro* and *in vivo*, hypochlorous acid (HOCl) is probably the most important (for a review see Weiss, 1989). Such oxidants can induce oxidation/decarboxylation and peroxidation of membrane amino acids and phospholipids respectively, generating chemotactic peptides and toxic aldehydes (Fantone and Ward, 1982; Rosen *et al.*, 1995; Anderson *et al.*, 1997).

Granule enzymes have broad substrate specificity with the capability to degrade almost all components of the extracellular matrix (Dallegri and Ottonello, 1997). Strongly cationic proteins such as defensins, serine proteinases, lysozyme and bactericidal/permeability increasing factor can severely disturb cell surface charge and enzyme function leading to membrane permeabilisation (Henson and Johnston, 1987).

However, there are two recurrent themes central to the pathogenesis of cellular injury. Firstly, that neither ROS nor proteolytic enzymes alone completely explain the destructive potential of neutrophil secretory products *in vivo* (Welbourn *et al.*, 1991). There is significant co-operation and synergy between different agents to optimise cytotoxic activity. Perhaps the best studied example of this synergy is the inactivation of human α-1 proteinase inhibitor (α-1PI; the most abundant high affinity serine proteinase inhibitor) by oxidation of an active site methionine residue by H_2O_2, HOCl and MMP's. This reduces the association rate between α-1PI and elastase 2000-fold, thereby facilitating elastolytic activity (Weiss, 1989; Dallegri and Ottonello, 1997).

The fact that many toxic neutrophil secretory products are rapidly neutralised by the presence of often saturating levels of their respective antioxidants and antiproteases introduces the second critical feature necessary for cytotoxic activity; the need to avoid or exclude inhibitors. This is primarily achieved through creation of a peri-cellular micro-environment at the point of apposition with the target cell that may stoichiometrically exclude antiproteases and/or, through tightly focussed respiratory burst activity, result in such high concentrations of proteases and ROS that antiprotease/antioxidant defences are overwhelmed. Clearly, neutrophil priming, sequestration of neutrophils in the microvasculature and adhesion molecule engagement will only serve to exacerbate the situation (Owen *et al.*, 1995; Liou and Campbell, 1996; Dallegri and Ottonello, 1997; Owen and Campbell, 1999).

This synergistic co-operation between oxidants and proteases in the pathogenesis of host tissue injury is summarised in Figure 1.1.

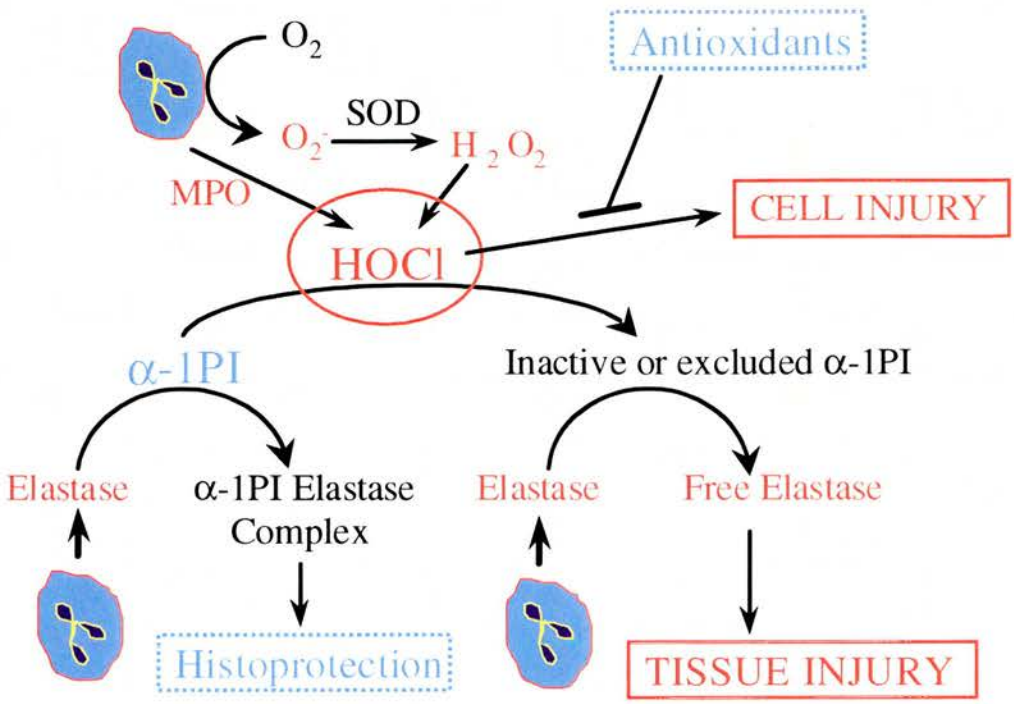


Figure 1.1 Schematic representation of the interaction of neutrophil secretory products and their inhibitors in the pathogenesis of host tissue injury

Stimulation of respiratory burst activity leads to secretion of reactive oxygen species (e.g. H_2O_2), proteolytic granule enzymes (e.g. neutrophil elastase) and cationic proteins. Inappropriate or overexuberant neutrophil activation in the face of inadequate or physically excluded inhibitors (anti-oxidants and proteinase inhibitors e.g. glutathione and α-1PI) can result in host tissue injury. See text for further details.

Neutrophils with a characteristic multilobulated nucleus are depicted in blue.

O_2^-: superoxide anions, SOD: superoxide dismutase, MPO: myeloperoxidase, HOCl: hypochlorous acid, α-1PI: α-1-Proteinase Inhibitor.

After Dallegri and Ottonello (1997)

Failure to terminate the inflammatory response, with continued recruitment of neutrophils or inadequate clearance of effete neutrophils will similarly increase the likelihood of unsolicited neutrophil-mediated tissue injury. To this end, low pH and high concentrations of cations and hypochlorous acid (all features of inflamed tissue) can inhibit macrophage uptake of apoptotic neutrophils *in vitro* (Haslett, 1997).

1.7 WHAT ROLE FOR THE NEUTROPHIL IN EQUINE COPD ?

Identification of a neutrophilic bronchiolitis as the archetypal pathological feature of equine COPD (Nicholls, 1978; Winder and Von Fellenberg, 1988; Kaup *et al.*, 1990a) and the generally consistent finding of elevated numbers or proportions of neutrophils in the tracheal secretions or BALF of clinically affected animals (Nuytten *et al.*, 1983; Bracher *et al.*, 1991; Vrins *et al.*, 1991; Dixon *et al.*, 1995c) provides strong circumstantial evidence for a causative role for neutrophils in disease pathogenesis. In a prospective clinical and lung biopsy histopathology study, Naylor *et al.* (1992) reported that bronchiolar neutrophil infiltration showed the strongest correlation with clinical assessment of disease severity.

Although tracheal secretion and BALF neutrophil ratios tend to be higher in more severely affected animals (Bracher *et al.*, 1991; Vrins *et al.*, 1991) and neutrophils are consistently recruited to the airways after at least 5 h of experimental challenge (Derksen *et al.*, 1985b; McGorum *et al.* 1993d), some studies indicate that neutrophils recruitment is not a prerequisite for airway dysfunction. In some susceptible animals, airway dysfunction is detectable prior to significant pulmonary neutrophil accumulation (Fairbairn *et al.*, 1993). Also intratracheal delivery of *F. rectivirgula* antigen to both COPD-susceptible and control ponies, despite increasing BALF neutrophil numbers in both groups, only induced airway obstruction in susceptible animals (Derksen *et al.*, 1988). A recent study of hay/straw challenged COPD-susceptible and healthy ponies clearly discriminated the groups on the basis of airway morphometry but not on neutrophil infiltration (Broadstone *et al.*, 1997). Although these data raise issues of sample size and the precise definition of control

and COPD-susceptible animals, it is clear that there is considerable heterogeneity in both the inflammatory and functional airway responses of susceptible horses to antigen exposure.

As previously noted the functional status as opposed to the mere accumulation of neutrophils is likely to be the critical determinant of their pathogenetic role. Preliminary studies suggest that basal (Marr *et al.*, 1997a) and agonist-stimulated (Olszewski *et al.*, 1999) superoxide anion generation is enhanced in peripheral blood neutrophils from COPD-susceptible horses following hay/straw challenge. Variable increases in BALF neutrophil buoyant density in COPD-affected horses has been interpreted as evidence of heterogeneous cell activation in the airways (Tremblay *et al.*, 1993). Olszewski and Laber (1993) reported increased ROS generation from tracheo-bronchial lavage cells compared to peripheral blood neutrophils from affected horses.

Increased levels of protease, MMP-9 type gelatinase, MMP-8 type collagenase and β-glucuronidase activity have been detected in the tracheal secretions of horses affected with COPD, suggestive of neutrophil degranulation in the airways. The levels of enzyme activity correlated with either the proportion of neutrophils in tracheal secretions or the severity of clinical signs (Von Fellenberg, 1987; Maisi *et al.*, 1994; Koivunen *et al.*, 1997a; Koivunen *et al.*, 1997b). Initial interest in the role of ENE stemmed from the erroneous conclusion that severely affected horses developed significant emphysema (reviewed in Section 1.1.4). On the assumption that unregulated neutrophil elastase activity would be central to the pathogenesis of the equine COPD, analogous to human COPD and pulmonary emphysema (Janoff, 1985), detailed investigation of ENE and its most abundant inhibitor (α-1PI) followed. However, more detailed pathological studies of equine COPD (Section 1.1.4) and the discovery that equine plasma contained not only greater amounts of α-1PI than human plasma but also at least 4 related glycoproteins (serine proteinase inhibitors, Spi 1-4) with multiple isoforms, rendered this hypothesis less tenable. Moreover, although equine Spi 1 was structurally similar to human α-1PI, containing an oxidation-sensitive methionine residue at its active site, this residue was substituted in the Spi 3 gene product (and probably also Spi 2 and 4) providing oxidation resistance (Patterson and Bell, 1989; Patterson *et al.*, 1991). A further

difference between the human and equine molecules is the absence of chemoattractant activity of the equine ENE/α-1PI complex for equine neutrophils in marked contrast to its human equivalent (Scudamore *et al.*, 1993).

A direct causal relationship between BALF neutrophil secretory products and airway obstruction/hyperresponsiveness has been studied *in vitro*. Although H_2O_2 modulates the contractility of isolated equine trachealis muscle (Olszewski *et al.*, 1995), co-incubation with activated neutrophils had no effect on cholinergic small airway contractile responses (Olszewski *et al.*, 1999).

Attempts to demonstrate a direct causative relationship between the presence of granulocytes or their secretory products in BALF and airway hyperresponsiveness *in vivo* have been unrewarding, even in human asthma, despite intensive study (Haley and Drazen, 1998). Undoubtedly the effects of airway inflammation such as mucus hypersecretion and plugging of small airways and airway wall thickening with concomitant narrowing of airway calibre will exacerbate airway obstruction and the consequences of hyperresponsiveness. However, the precise role of neutrophils in equine COPD is likely to remain elusive without use of experimental techniques such as neutrophil depletion or inhibition of specific neutrophil secretory products *in vivo*.

1.8 AIMS OF THE STUDY

A prerequisite to address the role of neutrophils in equine COPD is an understanding of the kinetics, functional status and eventual fate of neutrophils recruited to the lung. In light of current understanding of neutrophil biology and the pathogenesis of equine COPD the following aims were developed:

1. Definition of a purification method for equine neutrophils that minimised cell priming.
2. Characterization of priming and activation in equine peripheral blood neutrophils *in vitro* with a view to developing assays that could be used as an index of functional neutrophil priming *in vivo*.
3. Characterization of apoptosis in equine peripheral blood neutrophils *in vitro* that would permit recognition of neutrophil apoptosis *in vivo*.
4. Development of methods to facilitate assessment of the functional status of neutrophils recruited to the airways and harvested by BAL.
5. Assessment of the kinetics and function of peripheral blood and airspace neutrophils following experimental hay/straw challenge of COPD-susceptible horses.
6. Investigation of a potential role for neutrophil apoptosis and macrophage clearance in the resolution of airspace neutrophilia in equine COPD.

CHAPTER 2

MATERIALS AND METHODS

2.1 THE ISOLATION OF NEUTROPHILS FROM PERIPHERAL BLOOD

When performing *in vitro* studies on isolated neutrophils, a number of criteria must be optimised when selecting a purification method. Cell purity is paramount but the method must be rapid, maintain maximal cell viability and facilitate recovery of as many cells as possible from the initial sample to avoid the potential selection of a functionally biased cell subpopulation. Furthermore, it is well recognized that sub-optimal preparative techniques e.g. those causing cellular osmotic stress (Edashige *et al.*, 1993; Kitchen *et al.*, 1996b) or the use of reagents containing even trace amounts of LPS (Haslett *et al.*, 1985) may lead to cell priming and/or activation and thus significantly alter the results of subsequent functional studies.

Blood was drawn by venepuncture from healthy adult horses (either immediately post-mortem or from normal animals which records indicated had no history of respiratory disease) healthy human volunteers and anticoagulated with 1 ml of 3.8% sodium citrate per 10 ml of blood in 50 ml sterile polyethylene tubes. Aliquots of equine blood were submitted to the Clinical Pathology Laboratory, Royal (Dick) School of Veterinary Studies, Easter Bush Veterinary Centre for total and differential leucocyte counts. Total leucocyte counts were obtained using an automated haematology analyser (Baker/Biochem 9100, Biostat, Cheshire, UK) and a manual differential leucocyte was performed on slides stained automatically (Miles Haematech 2000, Bayer, Newbury, UK) with a modified Wright's trichrome stain.

2.1.1 PURIFICATION OF HUMAN PERIPHERAL BLOOD NEUTROPHILS

Human neutrophils were isolated on discontinuous hypotonic plasma/Percoll gradients exactly as detailed by Haslett *et al.* (1985) using sterile, LPS-free (<0.1

ng/ml LPS by the Chromagenix *Limulus* amoebocyte lysate assay) reagents and laboratory plastic-ware. This method has been documented to yield neutrophils that have minimal alterations in both their priming status (with respect to fMLP-stimulated superoxide anion generation) (Haslett *et al.*, 1985) and their resting cell morphology (less than 8% shape change as assessed by flow cytometry, [Cole *et al.*, 1995]). All procedures were carried out at room temperature, unless otherwise stated. The tubes of blood were centrifuged (300 x *g*, 20 min). The resulting supernatant of platelet-rich plasma was carefully aspirated and either centrifuged (2,500 x *g*, 20 min) to produce platelet-poor plasma (PPP), or used to prepare autologous serum by recalcification with 20 μM $CaCl_2$ at 37°C. Five ml of 6% dextran T-500 was added to the original cell pellet and 0.9% NaCl was then added to make a final volume of 50 ml. Following thorough but gentle mixing the contents were allowed to stand for 30-45 min to allow erythrocyte sedimentation.

The overlying leucocyte-rich plasma layer was carefully aspirated and centrifuged (300 x *g*, 6 min), and the mixed leucocyte cell pellet was resuspended in 2 ml PPP and transferred to a 15 ml polystyrene tube. The leucocytes from two 50 ml tubes could be used for a single gradient. A stock solution of 90% v/v Percoll was prepared in 0.9% NaCl (100% v/v Percoll: 0.9% NaCl, 9:1, v/v) and then further diluted with fresh autologous PPP to produce density gradients of 42% and 51% Percoll/PPP, which were maintained at 4°C until use. The resuspended cell pellet was underlayered sequentially with 2 ml of each of the 42% and 51% Percoll gradients, then centrifuged (275 x *g*, 10 min). Neutrophils were harvested from a broad band at the 42%/51% interface. Mononuclear cells remained in a narrower band at the upper PPP/42% interface.

Isolated neutrophils were then washed sequentially in PPP (500 x *g*, 6 min), PBS without Ca^{2+} and Mg^{2+} (PBS w/o; Sigma, Poole, Dorset), pH 7.4 (300 x *g*, 6 min) and PBS with Ca^{2+} and Mg^{2+}, pH 7.4, (PBS; Sigma, Poole, Dorset) (300 x *g*, 6 min). Cytocentrifuge preparations of isolated neutrophil populations were prepared by centrifuging (300 x *g*, 3 min) 100 μl of cells suspended in PBS w/o on to glass slides (Shandon Cytospin 3, Southern Instruments Ltd., Runcorn, UK). These were air-dried and fixed in methanol for 2 min. Cell purity was based on differential cell counts of 500 cells stained with a modified Wright-Giemsa stain (Diff-Quik)

examined under oil immersion (x 1000 magnification). Cell counts were determined using an Improved Neubauer haemocytometer (Fisher Scientific, Leicester, UK) and viability assessed by trypan blue exclusion.

2.1.2 PURIFICATION OF EQUINE PERIPHERAL BLOOD NEUTROPHILS

Three different methods for isolating neutrophils were compared, namely, two single-step techniques with discontinuous isotonic Percoll gradients (Pycock *et al.*, 1987; Jain *et al.*, 1990) and a modification of the method described above (Section 2.1.1) for isolating human neutrophils using discontinuous hypotonic plasma/Percoll gradients (Haslett *et al.*, 1985). In all methods, Percoll was maintained at 4°C until use, but all other procedures were performed at room temperature.

2.1.2.1 Method 1; Jain *et al.* (1990)

Three ml of citrated whole blood was underlayered with 2.5 ml 59% isotonic Percoll (100% Percoll: 10 x Hank's balanced salt solution [HBSS]; sterile de-ionised water, 5.9:1:3.1, v/v/v) and 2.5 ml 75% isotonic Percoll (100% Percoll: 10 x HBSS; sterile de-ionized water, 7.5:1:1.5, v/v/v) in a 15 ml polyethylene tube and centrifuged (400 x *g*, 20 min). Leucocytes were aspirated from the two interfaces; mononuclear cells from the upper (plasma/59% Percoll) and neutrophils from the lower (59%/75% Percoll) interface using a glass Pasteur pipette.

2.1.2.2 Method 2: Pycock *et al.* (1987)

Stock isotonic Percoll was prepared (100% Percoll: 10 x HBSS, 9:1, v/v). This stock (90% Percoll in HBSS) was subsequently diluted with 1 x HBSS to the appropriate concentration. Three ml of citrated whole blood was underlayered with 5 ml 70% Percoll and 5 ml 85% Percoll in a 15 ml polyethylene tube and centrifuged (400 x *g*, 20 min). Leucocytes were aspirated from the two interfaces; mononuclear cells from the upper (plasma/75% Percoll) and neutrophils from the lower (70%/85% Percoll) interface using a glass Pasteur pipette.

2.1.2.3 Method 3: Haslett *et al.* (1985)

This method was identical to that described for human blood above (Section 2.1.1), except that due to the high erythrocyte sedimentation rate of equine whole blood, the initial centrifugation and dextran sedimentation steps could be omitted. Whole citrated blood was allowed to sediment under gravity for 30 min at room temperature and the upper leucocyte-rich plasma layer was carefully aspirated and centrifuged (367 x *g*, 6 min). Percoll/PPP (41%/52% Percoll) gradients were prepared, layered beneath the resuspended mixed leucocyte pellet, centrifuged and cells harvested as described.

Isolated equine neutrophils were then washed sequentially in PPP (500 x *g*, 6 min), PBS w/o, pH 7.4 (300 x *g*, 6 min) and PBS, pH 7.4 (300 x *g*, 6 min). A more extensive wash protocol (four washes in PBS w/o/0.1% BSA, pH 7.4 and one wash in PBS/0.1% BSA, pH 7.4) was necessary in experiments investigating the mechanism of LPS priming in equine neutrophils, to reveal the serum dependence of the LPS effect (see Section 3.3.2.2).

Cytocentrifuge preparations of the isolated neutrophil populations were prepared, differential cell counts performed and cell number and viability determined as described in Section 2.1.1. Neutrophil recovery values were calculated using the differential cell counts generated by the Clinical Pathology Laboratory (Section 2.1). Table 2.1 shows the cell purity, viability and recovery rates for equine neutrophils isolated using the three different Percoll gradient methods outlined above.

Although the yield of neutrophils following the plasma/Percoll gradient step of the Haslett method was significantly lower than following the gradient step in the other two methods (49%, versus 64.5% for method 1 and 75% for method 2) cell losses during the subsequent wash protocol were significantly less (14% versus 38% method 1 and 40% for method 2). The cause of this discrepancy was not determined but may have reflected an enhanced adhesive capacity of the cells due to partial priming during the latter two isolation methods.

Hence, the method of Haslett and co-workers (1985) generated cells of consistent and high purity (98.2 ± 0.3%) and superior cell viability and recovery compared to the methods of Jain *et al.*, (1990) and Pycock *et al.*, (1987).

Isolation Method	Cell Purity (%) (Mean ± SEM)	Cell Viability (%) (Mean ± SEM)	Cell Recovery (%) (Mean ± SEM)
Jain *et al.*, 1990	96.3 ± 2.4	98.0 ± 0.5	26.5 ± 2.6
Pycock *et al.*, 1987	98.7 ± 0.6	97.7 ± 0.9	35.0 ± 4.7
Haslett *et al.*, 1985	98.2 ± 0.3	99.3 ± 0.1	43.8 ± 2.6

Table 2.1: Comparison of equine neutrophil populations isolated by three previously published techniques for equine and human neutrophils.
Data represent mean ± SEM of n = 4 -15 separate experiments.

This method was chosen as the technique of choice for the subsequent experiments, especially given the requirement for a uniform isolation method in experiments comparing the function of equine and human neutrophils. Using this method, isolated neutrophils were typically ready for use within 105 min of blood sampling. Variable numbers of eosinophils frequently contaminated the final preparations of human neutrophils (4.5 ± 1.6%, n = 5). However, a smaller number of eosinophils contaminated the equine preparations performed in parallel (1.2 ± 0.5%, n = 5). The equine eosinophils generally settled either below the 42/51% Percoll interface or with the erythrocyte pellet. This suggests a significant difference in density between these two equine cell types. Indeed, equine neutrophil and eosinophil populations of high purity (>99%) can be resolved on simple discontinuous gradients (Foster *et al.*, 1992) whereas subsequent immunoselection techniques are necessary to achieve similar results with human eosinophils (Stern *et al.*, 1992).
The light microscopic appearance of freshly isolated equine and human neutrophils is shown in Figure 2.1a,b.

a: HUMAN

b: EQUINE

Figure 2.1a,b: Light microscopic appearance of freshly isolated human and equine peripheral blood neutrophils

Human and equine peripheral blood neutrophils were isolated as described in Sections 2.1.1 and 2.1.2.3, respectively.

a: Photomicrograph of freshly isolated human neutrophils (magnification x 1480)

b: Photomicrograph of freshly isolated equine neutrophils (magnification x 1480)

Under the conditions of this preparative technique, the individual lobulated segments of human neutrophils were generally more rounded and both the individual lobes and their fine interconnecting chromatin strands were more clearly defined than those in equine neutrophils.

The method of Haslett *et al.* (1985) has been reported to induce minimal priming and shape change of human neutrophils (Condliffe *et al.*, 1996). In 12 sequential neutrophil isolations from different horses, basal shape change assessed by light microscopy (see Section 2.3.1.1) was confirmed to be extremely low at 2.9 ± 1.3%. The ability of this isolation method to produce equine neutrophils that were minimally primed or activated was confirmed in subsequent experiments (see Section 3.2.1) which demonstrated little if any basal or fMLP-stimulated respiratory burst activity in freshly isolated cells.

2.2 ASSESSMENT OF NEUTROPHIL RESPIRATORY BURST ACTIVITY

The increase in NADPH oxidase activity and ROS generation that underlies the respiratory burst of activated neutrophils was assessed by a chemiluminescence (CL) microtitre plate-based method (ML3000 Microtitre Plate Luminometer, Dynex Technologies Ltd., Billingshurst, UK). This method permitted collection of kinetic data from multiple samples in parallel (Blair *et al.*, 1988) and, by addition of different luminigenic probes to mixed cell populations in BALF samples, allowed differentiation of the neutrophil CL signal from that of other cell types.

2.2.1 CHEMILUMINESCENCE MEASUREMENT OF RESPIRATORY BURST ACTIVITY

The conditions selected for the CL assay were based on those recommended by Benbarek *et al.* (1996) and the luminometer manufacturer (Phagocyte Chemiluminescence, Luminescence Application Note, Dynex Technologies Ltd., Billingshurst, W. Sussex); hence the luminigenic probes lucigenin and luminol were used at final concentrations of 0.25 mM and 1mM respectively and all assays were

conducted at physiological pH (7.4) at 37°C. It is important to note however, that the normal physiological temperature for the horse is 37.8°C and not 36.8°C as in humans. Although CL of human and equine neutrophils has been shown to be temperature dependent (Washburn *et al.*, 1982; Allen, 1986; Benbarek *et al.*, 1996), no difference in the CL response of canine granulocytes has been demonstrated over the range 37 - 39°C (Burgener *et al.*, 1998). Hence, 37°C was chosen for both equine and human cells as a compromise to allow direct comparative studies between horse and human neutrophil function.

2.2.1.1 Preparation of luminigenic probes

Lucigenin: 6.3 mg of lucigenin (bis-N-methylacridinium nitrate) was dissolved in 50 ml PBS/0.1% BSA, pH 7.4 to give a final concentration of 0.25 mM. Fresh lucigenin was prepared daily and stored in the dark at 37°C until use.

Luminol: Stock luminol (10 mM) was prepared by dissolving 88.6 mg luminol (3-aminophthalhydrazide) in 5 ml 0.1 M NaOH, then adding PBS/0.1% BSA, pH 7.4 to make up to 50 ml. Luminol was stored in 1 ml aliquots at -20°C until use. Once thawed, stock luminol was diluted to 1 mM with PBS/0.1% BSA, pH 7.4 and equilibrated in the dark at 37°C.

2.2.1.2 Chemiluminescence assay

Lucigenin (0.25 mM) or luminol (1 mM) was added (100 µl) to each microtitre plate well and allowed to equilibrate at 37°C for 15 min prior to the addition of 80 µl of freshly isolated cells. Following a further 5 min incubation period, basal CL was recorded over 12 min to ensure that the cells were not basally activated (stable basal peak CL <0.005 Relative Light Units - RLU). Cells were discarded if basally activated. After pre-incubation, 20 µl of buffer or stimulus was added to triplicate wells and CL recorded at regular intervals over an appropriate time course. The CL response to fMLP (0.01 nM - 1 µM), zymosan-activated plasma (ZAP, 10% v/v), PMA (0.01 ng - 1 µg/ml), LTB_4 (0.01 nM - 1 µM), hrIL-8 (10 - 100 ng/ml), LPS (0.1 ng - 10 µg/ml), PAF (0.1 nM - 1 µM), hrTNFα (0.01 - 100 ng/ml) and erTNFα (0.1 - 1000 pg/ml) was assessed.

2.2.1.3 Data collection and analysis

Data were recorded and analysed on-line (Cellular Chemiluminescence, Dynex Technologies Ltd., Billingshurst, W. Sussex) to produce mean peak and integral (area under the curve) CL values in arbitrary RLU and nodal time [the time required for a set number of neutrophils to reach peak CL velocity (Allen, 1986)] data. CL values measured from triplicate blank wells (luminigenic probe and buffer only) were subtracted from values measured for all test samples.

CL data may be analysed using any of the above parameters (integral or peak CL, nodal time) or initial rate of increase in CL velocity (slope analysis) when neither neutrophils nor substrate (luminigenic probe) are rate limiting and reaction kinetics approximate zero-order (Allen, 1986). The kinetic geometry of the CL response varies with the concentration of a stimulus and to a lesser extent with the number of neutrophils (Allen, 1986; Lieberman *et al.*, 1996), (see Figure 2.2a). Hence, using a constant number of cells, CL was monitored over sufficient time to observe both the onset and complete decay of the response, to avoid missing a delayed CL peak or indeed a biphasic response as has been reported by certain groups for luminol-dependent CL (Lum-DCL) responses of human neutrophils to fMLP (Dahlgren *et al.*, 1985) and low concentrations of PMA (Allen, 1986). Integral CL values provide a measure of cumulative CL activity and account for both the rate of increase and the peak CL velocity and is considered the most useful parameter for inter-assay comparisons (Blair *et al.*, 1988). Although the kinetic geometry of neutrophil CL responses to different stimuli varies considerably (compare PMA, Figure 2.2a and fMLP, Figure 3.9), integral and peak CL values for an individual stimulus are usually well correlated (Van Dyke and Van Dyke, 1986). Integral and peak CL values for equine neutrophils in response to the agonists used routinely in these studies were highly correlated (fMLP + serum +/- LPS, $r = 0.97$, Figure 2.2b; PMA, $r = 0.82$; ZAP, $r = 0.83$, $p<0.0001$). Consequently, the integral CL value was chosen for data representation as it allowed better characterization of the response of equine neutrophils to a particular stimulus. Henceforth, all CL data are presented as integral values unless otherwise stated.

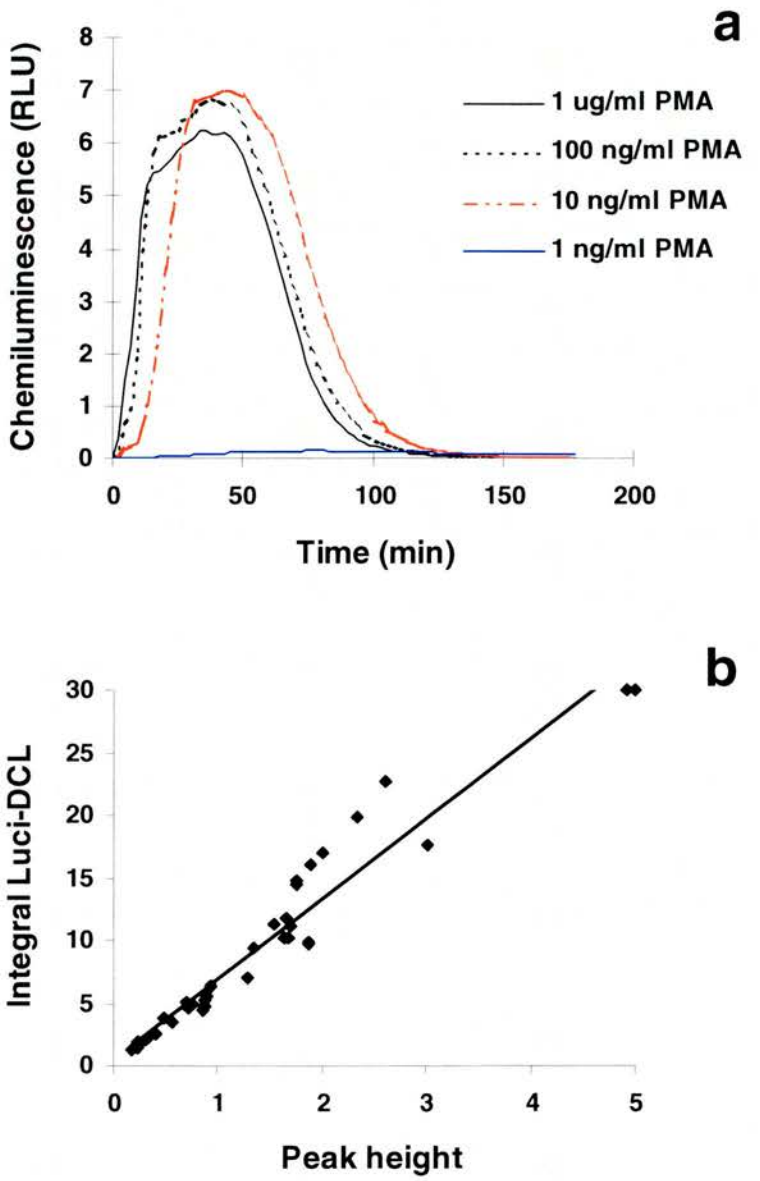


Figure 2.2a,b: Assessment of equine neutrophil respiratory burst activity by Lucigenin-dependent chemiluminescence (Luci-DCL)

a: Concentration-dependence of PMA-stimulated Luci-DCL kinetics. Neutrophils (1×10^6) were stimulated with 1 μg/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml PMA and Luci-DCL was recorded every 2.25 min (80 cycles) over 180 min. Nodal times were 34.0, 38.6, 45.3 and 76.8 min respectively. Values represent the mean of triplicate samples from a representative experiment of five that gave similar results.

b: Correlation of integral and peak Luci-DCL values. Neutrophils (1×10^6) were incubated with PBS or LPS (0.01, 0.1, 1, 10 & 100 ng/ml) in the presence of serum for 60 min prior to stimulation with fMLP (1 μM). Luci-DCL (RLU) was recorded every 10 s (30 cycles) over 5 min. $r = 0.97$. Pooled data from 6 experiments each performed in triplicate.

2.2.2 EFFECT OF CELL NUMBER ON LUCIGENIN-DEPENDENT CHEMILUMINESCENCE (Luci-DCL)

Although the initial velocity of CL generation immediately post-stimulation is independent of neutrophil number, the peak and hence integral values of luminigenic probe-dependent neutrophil CL are proportional to the number of cells used (Allen, 1986). This was confirmed using PMA-stimulated (10 ng/ml) Luci-DCL responses measured over 180 min with increasing numbers of equine neutrophils, where the intensity of Luci-DCL was shown to be dependent on cell number (Figure 2.3). A weak CL response was detectable with as few as 50,000 neutrophils per well (0.25 x 10^6 cells/ml) and this response increased up to 1 x 10^6 cells (5 x 10^6 cells/ml) but both integral and peak values diminished at high cell concentrations e.g. 5 x 10^6 cells (25 x 10^6 cells/ml); see Figure 2.3. A similar effect was recognized by Benbarek *et al.* (1996) where the use of very high cell numbers was also associated with a diminution of CL values, even when a greater assay volume was used. Subsequently all assays were performed using 1 x 10^6 neutrophils/well (equivalent to 5 x 10^6 cells/ml).

2.2.3 NEUTROPHIL PRIMING ASSAYS

In experiments designed to investigate priming of equine neutrophils, cells resuspended in PBS were incubated in a final volume of 1 ml in 2 ml round-bottomed Eppendorf tubes in a shaking waterbath at 37°C for 5 min prior to the addition of known human priming agents at predetermined optimal times: LPS (0.1 ng - 1 μg/ml, 30, 60 or 90 min) in the presence and absence of 1% heat-inactivated autologous serum, PAF (0.1 - 10 μM, 10 min) and hrTNFα (0.01 - 100 ng/ml, 30 min), erTNFα (0.1 – 1000 pg/ml, 30 min) or hrIL-8 (10 - 100 ng/ml, 30 min). In initial priming experiments, the concentration of hrTNFα used was based on units of cytotoxic activity (U/ml). Subsequently human and equine rTNFα were compared on a weight of protein basis. During incubation periods greater than 15 min, the Eppendorf tubes were removed briefly every 15 min and gently tapped to ensure that the cells remained fully suspended throughout the priming period.

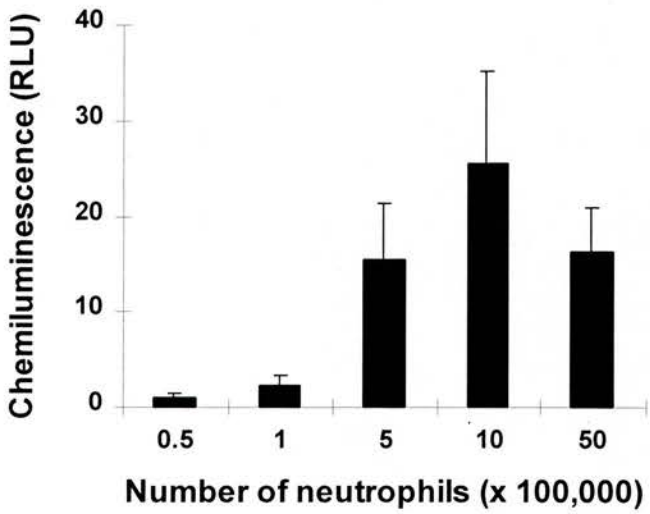


Figure 2.3: Effect of cell number on PMA-stimulated Luci-DCL in equine neutrophils. Integral Luci-DCL values from increasing numbers of neutrophils in response to 10 ng/ml PMA measured over 180 min. Values represent mean ± SEM of 3 separate experiments each performed in triplicate.

The neutrophil concentration was adjusted in these experiments to maintain the same number of cells (1×10^6) in the final CL assay. At the end of the incubation period, cells were transferred to the luminometer microtitre plate and the assay performed as detailed above.

2.2.4 OPTIMISATION OF CHEMILUMINESCENCE TIME COURSES FOR INDIVIDUAL AGONISTS

Following the initial experiments described above to determine the optimal priming conditions and the effect of cell number and agonist concentration on Luci-DCL responses, the time course of the CL signal stimulated by individual secretagogue agonists was determined. Subsequently, CL was recorded for 5 min with fMLP, 30 min for PAF, LPS and human and equine rTNF-α and for 90 min with all other agonists.

To ensure that the post-peak decline in the CL response to a stimulus was not due to cell death, neutrophil viability was determined after a 180 min time course by their ability to exclude trypan blue. The results presented in Table 2.2 confirm that cell viability was maintained over 180 min and was unaffected by stimulation with either ZAP or PMA.

2.3 ASSESSMENT OF NEUTROPHIL SHAPE CHANGE

Stimulation of neutrophils by priming or secretagogue/chemotactic agents initiates or enhances changes in cell shape; this includes ruffling of the plasma membrane, pseudopodia extrusion and polarization, and is associated with re-structuring of the cytoskeleton, particularly F-actin (Fernandez-Segura *et al.*, 1995). This response is thought to represent frustrated phagocytosis and is considered to be a very sensitive indicator of priming (Haslett *et al.*, 1985).

Stimulus	Cell Viability (%) (Mean ± SEM)	Number of experiments
Buffer	97.6 ± 0.6	6
ZAP (10% v/v)	94.9 ± 1.8	7
PMA (0.1 ng/ml)	97.7 ± 1.3	2
PMA (1 ng/ml)	96.4 ± 1.29	3
PMA (10 ng/ml)	97.1 ± 1.0	5
PMA (100 ng/ml)	92.6 ± 2.7	4
PMA (1μg/ml)	96.2 ± 1.2	2

Table 2.2: Viability of equine neutrophils after 180 min CL time course.
Neutrophils were stimulated with buffer, ZAP (10% v/v) or PMA (0.1 – 1000 ng/ml) and Luci-DCL recorded over 180 min. Cells were harvested from the microtitre plate and viability assessed by trypan blue exclusion. The viability of freshly isolated cells was 99.3 ± 0.1% (Table 2.1).

2.3.1 SHAPE CHANGE ASSAY

Neutrophils (2 x 10^6 / 900 µl PBS in 2 ml round-bottomed Eppendorf tubes) were incubated at 37°C for 5 min in a shaking waterbath. Duplicate samples were then incubated for 10 min with 100 µl buffer or agonist. Incubations were terminated by the addition of an equal volume of 2.5% glutaraldehyde. In priming experiments, cells were incubated with priming agents as detailed in Section 2.2.3, prior to the addition of 100 µl buffer or agonist.

Shape change was quantified by light microscopy and/or flow cytometry and cell ultrastructure and surface morphology were assessed with transmission (TEM) and scanning (SEM) electron microscopy, respectively.

2.3.1.1 Light microscopy

At least 200 neutrophils were examined by phase contrast microscopy at x 400 on a haemocytometer and shape change quantified as the percentage of cells extruding more than one small pseudopodium (see Figure 2.4) as previously described by (Kitchen *et al.*, 1996a).

2.3.1.2 Flow cytometry

Shape change was quantified by flow cytometry (EPICS Profile II, Coulter Electronics, Luton, Bedfordshire, UK) using a minor modification of the method described by (Cole *et al.*, 1995). Ten thousand events were analysed by measurement of forward angle light scatter. A gate was set to include the unimodal distribution of unstimulated (buffer only) cells and shape change was quantified as the percentage of cells lying outside the gate following stimulation (see Figure 2.4).

2.3.1.3 Comparison of methods

In previous studies, shape change measured in human neutrophils by flow cytometry correlated closely with values obtained by direct light microscopic assessment, with the exception that the flow cytometric method slightly over-estimated the extent of basal shape change and slightly under-estimated the proportion of cells in highly activated cell populations (Cole *et al.*, 1995).

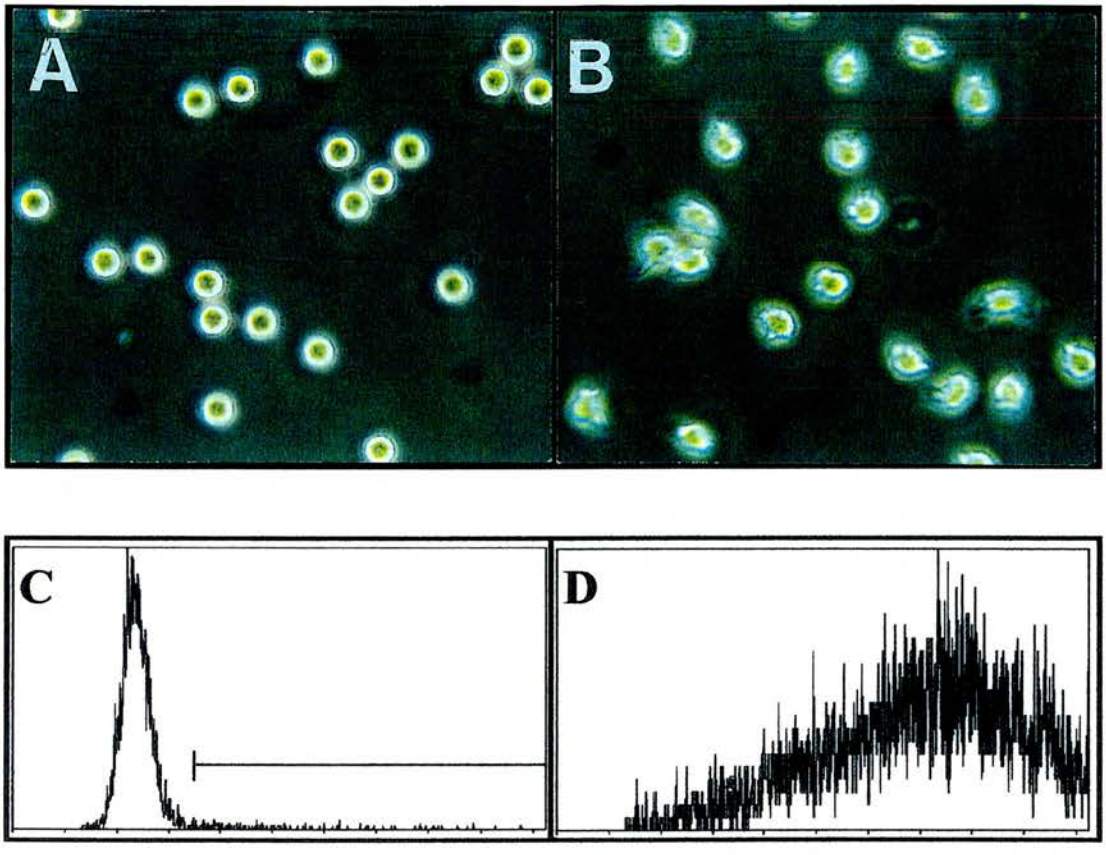


Figure 2.4: Assessment of equine neutrophil shape change.

Polarized light microscopic appearance (panels **A** & **B**, magnification x 500) and flow cytometer frequency histograms (panels **C** & **D**) of glutaraldehyde-fixed equine neutrophils following incubation (10 min, 37°C) with buffer (panels **A** & **C**) or 10% v/v ZAP (panels **B** & **D**). Horizontal bar (panel **C**) indicates position of the gate delineating non-shape changed cells. Using this analysis 97.4% of the cell population in panel **D** was considered to be shape changed following activation by ZAP (10% v/v). Representative experiment of 10.

Light microscopy data for equine neutrophils were strongly correlated with those obtained by flow cytometry ($r = 0.94$, $p<0.0001$, Figure 2.5). All subsequent equine neutrophil shape change experiments were analysed by flow cytometry.

2.3.2 ULTRASTRUCTURAL MORPHOLOGY

The ultrastructural morphology of populations of quiescent (incubated in PBS, 90 min, 37°C), primed (100 ng/ml LPS, 0.1% heat-inactivated autologous serum, 90 min) or activated neutrophils (ZAP 5% v/v, 10 min) was examined by transmission (TEM) and scanning electron microscopy (SEM).

Cells were pelleted (230 x *g*, 6 min), resuspended and fixed in 1 ml sodium cacodylate buffer (0.1M, pH 7.4)/2.5% glutaraldehyde then re-pelleted (250 x *g*, 6 min). Mr S. Mitchell of the Electron Microscopy Unit, Department of Preclinical Veterinary Science, Royal (Dick) School of Veterinary Studies kindly performed subsequent processing.

The ultrastructural morphology of apoptotic equine neutrophils was assessed after 20 h in culture (see Section 2.6.1). Cells were harvested (see Section 2.6.2) and prepared for TEM.

2.3.2.1 Transmission electron microscopy

After post-fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 45 min, samples were washed three times in distilled water prior to dehydration in acetone and infiltration in a mixture of araldite CY212 (Agar Scientific Ltd., Stansted, UK) and dodecenyl succinic anhydride (DDSA - Agar Scientific Ltd.). At the final stage of infiltration and embedding, an accelerator containing 67% dibutylphthalate (Agar Scientific Ltd.) and 33% dimethylaminomethylene-30 (Agar Scientific Ltd.) was added to the araldite and DDSA mixture to harden the samples. One μm thick specimens were then cut on an ultramicrotome (Reichert Ultramicrotome OMU4, Leica UK Ltd., Milton Keynes, UK) utilising a glass knife. Sections were then stained with toluidine blue and examined under light microscopy to select appropriate areas to obtain sections for TEM examination. Sixty nm thick sections were cut and mounted on a copper grid, stained with uranyl acetate and lead

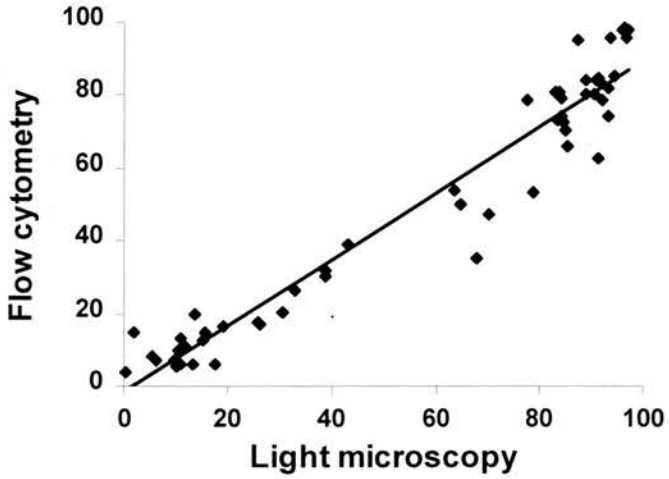


Figure 2.5: Comparison of light microscopy and flow cytometry for measurement of shape change in equine neutrophils

Cells were incubated with buffer or ZAP (10 and 1% v/v), PMA (100, 10 and 1 ng/ml), fMLP (1 μM and 100 nM), PAF (100 nM) and LTB_4 (100 and 10 nM) for 10 min. Cells were fixed with 2.5% glutaraldehyde and shape change quantified by light microscopy and flow cytometry as described above. ($r = 0.94$, $p<0.0001$). Pooled data from 54 assays (values represent mean of duplicate samples) from 4 separate experiments.

citrate (LKB Ultrastainer,Leica UK Ltd.) and examined and imaged in a TEM (Philips EM400, Philips Electron Optics, Eindhoven, The Netherlands).

2.3.2.2 Scanning Electron microscopy

After impregnation with three changes of a combination of purified paraffin and plastic polymers (Paraplast Plus, Sherwood Medical, Athy, Eire), the specimens were embedded in this material. Ten μm thick sections were cut from each block and mounted on aluminium stubs (Pin Stub, Emitech Ltd., Ashford, Kent) using conductive carbon cement (Leit-c, Neubauer Chemikalien, Munster, Germany) and coated with gold-palladium (Gold/Palladium target, Emitech Ltd) in a sputter coater (SC500 Sputter Coater, Emscope Laboratories Ltd., Ashford, Kent). Samples were examined and imaged in a SEM (Philips EM505, Philips Electron Optics) operated at 30.2 kV.

2.4 ASSESSMENT OF NEUTROPHIL CHEMOTAXIS

Neutrophils resuspended in PBS (3 x 10^6 cells/ml) were incubated with 1% serum for 90 min in the presence or absence of LPS (final concentration 1 μg/ml) and then chemotaxis assayed in a Neuroprobe™ 96-well chemotaxis chamber (Porvair Filtronics Ltd, UK) incorporating a 5 μm pore size polycarbonate filter (10 μm thick) (Falk *et al.*, 1982; Harvath and Leonard, 1982). PBS (Sigma, control), fMLP (0.01 - 1000 nM) or ZAP (10% v/v) was placed into the lower wells (35 μl) and 225 μl of neutrophil suspension added to the upper wells. The chamber was incubated in a humidified 5% CO_2 atmosphere at 37°C for 90 min. The filter was then removed, the upper surface scraped with a cell scraper, washed with 0.9% saline, air dried, fixed in methanol and stained with Diff-Quik (Falk *et al.*, 1982; Harvath and Leonard, 1982). Cell migration into, and retention within the filter was quantified by measuring the optical density of each well footprint at 500 nm in a MR 5000 plate reader (Dynex Technologies Ltd, Billingshurst, West Sussex, UK).

2.5 MEASUREMENT OF [^{3}H] fMLP BINDING TO NEUTROPHILS

The [^{3}H]fMLP binding assay was based on the method described by O'Flaherty *et al.* (1991). Equine and human neutrophils (12.5 x 10^6/ml) were pre-incubated for 90 min in PBS plus 1% serum in the presence or absence of 1 μg/ml LPS then diluted with an equal volume of ice-cold PBS containing 10 mM N-(2-hydroxymethyl) piperazine N'-2-ethanesulphonic acid (HEPES) buffer (pH 7.4), centrifuged (235 x *g*, 6 min) and resuspended at 5.26 x 10^6 cells/ml. 950 μl of cells (5 million) were carefully layered onto 400 μl of silicone oil in 1.5 ml Eppendorf tubes. [^{3}H]fMLP binding was performed by incubating cells, in triplicate, in the above buffer at 4°C for 60 min with 0.0632, 0.2, 0.632 or 2 nM [^{3}H]fMLP alone or 2 nM [^{3}H]fMLP with sufficient fMLP to make up final ligand concentrations of 2 - 6320 nM in half log dilution steps. Incubations were terminated by centrifugation (11,300 x *g*, 2 min, 4°C) through the silicone oil cushion. Aliquots (200 μl) of the supernatant were transferred to scintillation vials. Following aspiration of the residual supernatant and silicone oil, the tips of the Eppendorf tubes containing the cell pellet were cut off and transferred to scintillation vials. The pellets were incubated with 500 μl methanol for 20 min. Four ml of scintillation fluid was added to each sample prior to scintillation counting. Data underwent Scatchard analysis using LIGAND software (Munson and Rodbard, 1980) where the number of fMLP molecules bound per neutrophil was calculated using Avogadro's constant and the number of picomoles of fMLP bound per 5 x 10^6 cells.

2.6 ASSESSMENT OF EQUINE NEUTROPHIL APOPTOSIS *IN VITRO*

2.6.1 NEUTROPHIL CULTURE

Freshly isolated equine and human neutrophils were resuspended (5 x 10^6/ml) in "Monofeed" (MF) (Iscove's modified Dulbecco's medium supplemented with 10% autologous serum - prepared by recalcification of platelet-rich plasma - and 100 U/ml penicillin and streptomycin). Neutrophils (625,000 cells/135 μl) were aged in culture

in polypropylene flat-bottomed flexiwells at 37°C in an humidified 5% CO_2 atmosphere for up to 36 h with either MF (control) or test reagent (15 µl). The effect of culture with LPS (0.1 ng - 10 µg/ml), hrGM-CSF (500 U/ml), PAF (1 µM), fMLP (1 µM), PMA (0.1 ng - 1 µg/ml), hrTNFα (0.01 - 100 ng/ml), erTNFα (0.1 pg - 1 ng/ml), zymosan activated serum - a biological source of C5a (ZAS - 10% v/v) or dexamethasone (DEX; 0.1 nM -1 µM) were investigated. Also the effect of phagocytosis of opsonised ovine erythrocytes (OsRBC) was investigated by culture of these particles with neutrophils at a ratio of 3:1.

2.6.2 MORPHOLOGICAL ASSESSMENT OF NEUTROPHIL APOPTOSIS

Neutrophil apoptosis was assessed morphologically in accordance with the method of Savill *et al.* (1989) for human neutrophils. At appropriate time points (typically after 8 and 20 h in culture) cells were gently resuspended and 100 µl (approximately 5×10^5 neutrophils) harvested from each well using a cell saver pipette tip, cytocentrifuged (300 x *g*, 3 min) and the resulting slide preparations air dried, fixed with methanol and stained with Diff Quik. Use of cell saver pipette tips during harvesting results in less cell trauma and associated disruption of light microscopic morphology compared to standard tips (Dr. J. Murray, personal communication) and allowed recovery of the vast majority of cells. Cell viability was assessed in parallel by trypan blue exclusion. Cell morphology was examined by x 1000 oil immersion light microscopy and apoptotic neutrophils defined as cells containing one or more darkly staining pyknotic nuclear remnants (see Figure 4.1). For each condition examined, 500 cells were counted on triplicate slides with the observer blinded to the assay conditions.

2.6.2.1 Assessment of cell adhesion

To ensure that adhesion of non-apoptotic cells to the culture vessel did not lead to an artefactually increased proportion of apoptotic cells being harvested from the flexiwells, cell adhesion was assessed by methylene blue staining. Following resuspension and complete aspiration of well contents, residual cells were fixed with neutral buffered formalin (100 µl, 4% in PBS, 1 h, room temperature). Plates were

centrifuged (235 x *g*, 5 min), the formalin discarded and then stained (30 min) with pre-filtered 1% methylene blue. Excess dye was washed away with distilled water, the cells lysed with 100 mM HCl and the optical density of wells was measured at 630 nm in a MR 5000 plate reader (Dynex Technologies Ltd.). This showed that only a very small proportion of cells were not recovered and failed to demonstrate any significant difference in rates of adhesion between different culture conditions (data not shown).

2.6.3 EFFECT OF CELL DENSITY ON THE RATE OF CONSTITUTIVE EQUINE NEUTROPHIL APOPTOSIS

As the rate of constitutive human neutrophil apoptosis *in vitro* has been shown to be modulated by cell density (Hannah *et al.*, 1998), this effect was investigated for equine neutrophils in the culture system described above. Cells were cultured for 20 h at 2.5, 5, 7.5 and 10 x 10^6/ml and the rates of constitutive apoptosis compared. As in human cells, the rate of apoptosis in equine neutrophils cultured for 20 h was inversely proportional to the cell density (see Figure 2.6), although this trend was not statistically significant. All subsequent experiments were performed with cells resuspended at 5 x 10^6/ml.

2.6.4 ASSESSMENT OF EQUINE NEUTROPHIL APOPTOSIS BY CHROMATIN FRAGMENTATION

The chromatin of cells undergoing apoptosis fragments in a characteristic internucleosomal pattern that is recognized as a distinctive "ladder" on electrophoresis of DNA. Cells (4 x 10^6) were aged in culture and harvested as detailed in Sections 2.6.1 and 2.6.2 and centrifuged (2,000 x *g*, 2 min). Cells were then lysed in 0.5 ml, 6 M guanidine hydrochloride containing 20 mM Tris (hydroxymethyl) aminomethane-Cl (Tris), pH 8.0 and 0.1% N-lauryl sarcosine. An equal volume of Tris-Cl pH 8.0-buffered phenol/$CHCl_3$ (1:1) was added and the mixture inverted 6 times. The organic phenol/$CHCl_3$ phase was then spun out (11,300 x *g*, 10 min) at room temperature. The upper DNA-containing phase was

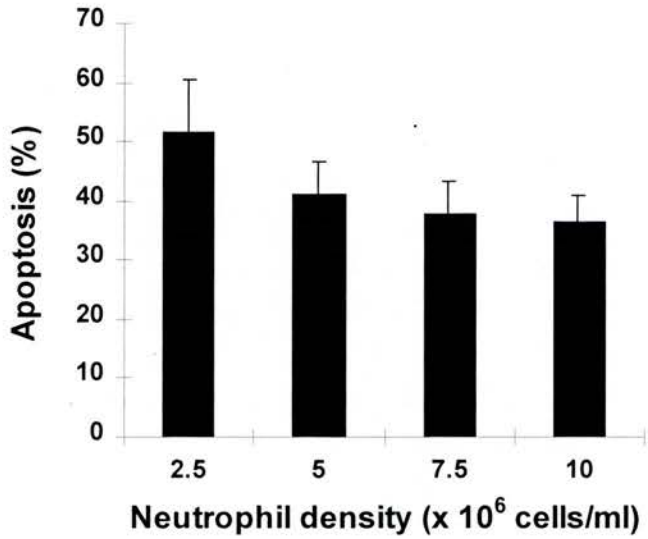


Figure 2.6. The effect of cell density on constitutive apoptosis in equine neutrophils
Cells were cultured for 20 h at the densities shown. Cells were resuspended and percent apoptosis assessed morphologically on Diff Quik-stained cytocentrifuge preparations. Values represent mean ± SEM of 5 separate experiments, each performed in triplicate.

removed, placed in a new microcentrifuge tube and precipitated from solution by the addition of an equal volume of propan-2-ol and 1/10 volume 3 M sodium acetate solution. The DNA was pelleted (11,300 x *g*, 5 min) and resuspended in 100 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA-Na, pH 8.0) containing 50 µg/ml RNase A. After incubation at 37°C for 15 min the DNA was loaded onto a 1.6% agarose gel and electrophoresed (100 volts, 2 h) through 0.5 x TBE Buffer (0.5 x TBE – 89 mM Tris, 89 mM boric acid, 5 mM EDTA) and then imaged by staining with 0.5 µg/ml ethidium bromide and photographed with a polaroid camera over an UV transilluminator. A 1 Kbp DNA ladder marker (Life Technologies, Paisley, UK) was also included.

2.6.5 ASSESSMENT OF EQUINE NEUTROPHIL APOPTOSIS BY ANNEXIN V BINDING

As cells become apoptotic, plasma membrane phospholipid asymmetry is lost such that phosphatidylserine becomes exposed on the outer leaflet of the membrane. This can be detected by the binding of Annexin V (Homburg *et al.*, 1995), a protein that binds to phospholipids especially phosphatidylserine, in a calcium-dependent manner. Neutrophils were harvested and incubated on ice for 10 min with FITC-labelled Annexin V diluted 1:4 in Annexin V Buffer. Preliminary experiments showed that the proportion of aged cells binding Annexin V was very similar, irrespective of whether samples were analysed immediately after cell harvest and staining or following subsequent fixation (3% paraformaldehyde/PBS, 10 min), washing (1000 x *g*, 2 min), resuspension in buffer (PBS/0.2% BSA/0.1% sodium azide) and overnight storage at 4°C (data not shown). Samples were analysed in a FACScan flow cytometer (Becton Dickinson, Oxford, UK). 10,000 events were analysed and a gate set to determine the percentage of Annexin V-FITC positive cells.

2.6.6 TERMINAL DEOXYNUCLEOTIDE TRANSFERASE MEDIATED UTP NICK END-LABELLING (TUNEL) OF APOPTOTIC EQUINE NEUTROPHILS

To allow simultaneous identification of nuclear condensation and DNA fragmentation in apoptotic neutrophils, the terminal 3'-OH groups of fragmented DNA were labelled *in situ* by TUNEL using a FragEL™ DNA Fragmentation Detection Kit (Calbiochem - Novabiochem, Nottingham, UK). Cytocentrifuge preparations of aged neutrophils were prepared on TESPA- (3-aminopropyltriethoxysilane) coated slides, fixed (4% formalin/PBS, 10 min), air dried (2 h) and stored at -70°C until use. Positive control specimens were prepared by incubation with 5 μg/ml DNase 1 at 37°C for 30 min. Labelled DNA was detected with DAB (3,3'-diaminobenzidine tetrahydrochloride) and slides counterstained with methyl green.

2.7 CLINICAL EVALUATION AND CHARACTERIZATION OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE-SUSCEPTIBLE HORSES

2.7.1 SUBJECTS

Six horses (two Thoroughbreds, one Thoroughbred-cross, two Cob crosses and one mixed breed), (4 geldings, 2 mares), median age 17.5 years (range 12 - 30), median body weight 518 kg (range 454 - 580) with a history of recurrent respiratory disease following exposure to mouldy hay and straw were used for the hay/straw challenge experiments.

2.7.2 HAY / STRAW CHALLENGE

During the study, horses were housed individually, in well ventilated stables (4.9 m x 4.0 m x 3.6 m high, open-fronted, Yorkshire boarding ventilation at the rear), bedded on wood shavings and fed haylage (ensiled grass) ad lib; henceforth termed

"controlled environment". All animals were treated with anthelmintics every 6 weeks and vaccinated against tetanus every 2 years.

Hay/straw challenge was performed in an enclosed, poorly ventilated (ridge ventilation only) stable (3.4 m x 3.6 m x 3.6 m high). Deep litter straw bedding was maintained in the stable for at least 2 weeks prior to any challenge. Poorly saved hay, that often had both a musty odour and evidence of mould contamination, was fed in a hay rack and on the floor. The hay was shaken out prior to the horse's entry and once during the challenge period. The same batch of poorly saved hay was used in all challenges.

A clinical scoring system was designed in an attempt to quantify clinical signs of respiratory disease prior to and following hay/straw challenge. Six parameters were evaluated on clinical examination combining categorical and continuous data, from which a total score out of 10 was computed, see Table 2.3.

Carotid arterial blood, collected with a 21g, 40 mm needle into heparinised syringes (Arterial Blood Sampler, Chiron Diagnostics, Halstead, UK), was stored on ice until analysed (maximum delay - 20 min) for PaO_2, $PaCO_2$ and blood pH in a Corning 238 Blood Gas Analyser (Ciba Corning Diagnostics Ltd., Halstead, UK).

Venous blood was collected as described previously (Section 2.1) for total and differential leucocyte counts and neutrophil isolation.

2.7.3 COLLECTION OF BRONCHOALVEOLAR LAVAGE FLUID (BALF)

BALF was collected transendoscopically via a 2 or 3 m video-endoscope (Olympus CF 200HL or Olympus XQ30, Olympus Keymed, Southend-on-Sea, UK). Horses were sedated with a combination of romifidine (30 μg/kg, i/v; Sedivet, Boehringer Ingleheim Ltd., Bracknell, UK) and butorphanol (20 μg/kg, i/v; Torbugesic, Willows Francis Veterinary, Crawley, UK) and further restrained with a nose twitch during BALF collection. Bronchoalveolar lavage (BAL) was performed by wedging the tip of the endoscope in the lobar bronchus of the accessory lobe of the right lung (Sweeney *et al.*, 1992b; Halliwell *et al.*, 1993), followed by rapid infusion of 300 ml (5 x 60ml) of pre-warmed (37°C) 0.9% NaCl through the biopsy channel of the endoscope, followed by a bolus of 30 ml of air; thereafter BALF was aspirated by

PARAMETER	OBSERVATION	SCORE
Cough[a]	Absent	0
	Present	1
Nasal discharge	Absent	0
	Present	1
Dyspnoea[b]	Absent	0
	Mild	1
	Moderate	2
	Severe	3
Respiratory rate[b] (breaths/min)	< 20	0
	20-30	1
	> 30	2
Auscultation - trachea[c]	Normal	0
	Abnormal (Fluid sounds)	1
Auscultation - lung fields[d]	Normal breath sounds	0
	Increased normal breath sounds	1
	Detectable adventitious[e] breath sounds	1
	Marked adventitious[e] breath sounds	2
TOTAL	**Maximum score**	**10**

Table 2.3 Clinical Scoring system adopted for systematic clinical examination

Notes: a: score 1 for either spontaneous cough or cough during induced hyperpnoea.

b: determined at rest prior to restraining horse.

c: auscultation over distal cervical trachea; categorised as normal breath sounds or evidence of accumulation of respiratory secretions.

d: determined at rest and during and after 2 min of hyperpnoea induced with a 20 L rebreathing bag.

e: wheezes and crackles

hand using a 60 ml syringe. Typically, this procedure was completed in 60 s. The BALF was stored in sterile 50 ml polypropylene tubes at room temperature until processed in the laboratory (maximum delay - 20 min).

In order to study the temporal cellular and biochemical changes in BALF following hay/straw challenge, a sequential BAL protocol in different lobes was developed. This was designed to minimise the effects of prior BAL, which has been shown to affect both BALF cell number and function in a number of species, including the horse (Cohen and Batra, 1980; Ohmann and Babiuk, 1986; Haley *et al.*, 1989; Sweeney *et al.*, 1994).

A baseline BAL was performed in the right accessory lobe at least 10 days prior to hay/straw challenge. The times and sites of BALF collection before and after challenge are shown in Table 2.4. Having entered the correct bronchus, the endoscope was advanced until wedged and the BAL performed as described above. A sham challenge protocol was performed (bronchoscopy and BAL only), to ensure that sequential BAL alone did not cause significant alterations in BALF cell numbers and proportion (6 horses) or BALF cell function as assessed by Luci- and Lum-DCL (3 horses).

2.7.4 SCORING OF TRACHEAL SECRETIONS

The volume of tracheal secretions was assessed and scored during endoscopic inspection at the level of the thoracic inlet prior to BAL. Secretions were scored semi-quantitatively on a scale of 0 -5 (see Table 2.5), using a modification of the system described by Dixon *et al.* (1995a).

BAL NUMBER	TIME AFTER START OF CHALLENGE	SITE[a]
BASELINE	≥ -10 d	right accessory (RB3)
1	+ 5 h	right accessory (RB3)
2	+ 24 h	left caudal (LB2, 1V)
3	+ 4 days	right caudal (RB2, 4L)[b]
4	+ 7 days	left caudal (LB2, 4L)[b,c]
5	+ 14 days	right accessory (RB3)

Table 2.4 Times and anatomic sites of BAL for sequential BAL protocol following hay / straw challenge

Names of bronchi are as described by Sweeney *et al.* (1992b).

Notes: a: letters and figures in brackets refer to nomenclature described by Sweeney *et al.* (1992b).

b: in one animal following challenge, these samples were collected from RB2 and LB2 respectively, using a 3m endoscope.

c: in two animals after challenge, BALF was collected from the left cranial bronchus (LB1).

VOLUME OF TRACHEAL SECRETIONS	SCORE
None[a]	0
A few flecks[a]	1
Multiple blobs	2
A complete stream <1/3 diameter of trachea	3
A complete stream >1/3 but <1/2 diameter of trachea	4
A complete stream > 1/2 diameter of trachea	5

Table 2.5: Semi-quantitative endoscopic scoring scale for tracheal secretions.

Note: a: scores of 0 and 1 were considered normal

2.7.5 CHALLENGE PROTOCOLS

All horses were confirmed to be free of pulmonary disease as assessed by clinical examination, arterial blood gas analyses and BALF cytology, after at least 8 weeks either at pasture or housed in a controlled environment. After a minimum of 10 days in a controlled environment, horses were confirmed to be susceptible to COPD by exposing them to either a 5 h or an overnight (8 h) hay/straw challenge, followed by clinical examination and BAL. Horses were defined as suffering from COPD if they developed clinical signs of airway obstruction (clinical score ≥ 3; see Table 2.3) associated with accumulation of neutrophils in the airspaces (BALF neutrophilia >5%) (McGorum *et al.*, 1993b). Horses were then either returned to pasture or housed in a controlled environment for at least 8 weeks prior to further study. Following a minimum period of 10 days after the baseline evaluation horses underwent hay/straw challenge for 5 h as described in Section 2.8.2, followed by sequential evaluations as detailed in Table 2.4. At each time point, a clinical examination was performed, arterial and venous blood samples were collected (Section 2.8.2) and bronchoscopy was performed with scoring of tracheal secretions (Section 2.8.4) and collection of BALF (Section 2.8.3).

2.8 ANALYSIS OF CELLULAR AND MOLECULAR COMPONENTS OF BRONCHOALVEOLAR LAVAGE FLUID (BALF)

2.8.1 PROCESSING OF BALF

After collection, all aliquots of BALF were pooled, the volume measured and the percentage recovery of the instilled fluid determined. Aliquots of raw BALF were taken for determination of total cell count, using an haemocytometer, cell viability by trypan blue exclusion and for determination of differential cell counts on Leishman's-stained cytocentrifuge preparations. After challenge, BALF often contained significant macroscopic amounts of mucus that markedly hindered initial attempts to isolate neutrophils from the BALF by density gradient centrifugation. In

this laboratory, BALF had traditionally been filtered through a single layer of cotton gauze to remove surfactant and mucus. However, residual mucus often remained after gauze filtration, so other methods of mucus removal were investigated. Biochemical dispersal of mucus by dithriothreitol (DTT) has been used to facilitate cytological interpretation of induced sputum samples from humans (Keatings and Barnes, 1997). The effect of DTT on equine BALF was assessed by incubation of DTT (0.01 - 1% w/v) with 2 ml aliquots of raw BALF from 3 animals for up to 60 min. Mucolysis was assessed visually to determine the optimal concentration that eliminated visible mucus flecks. Mucolysis was only observed at concentrations of DTT > 0.1%. Given that DTT is a strong reducing agent, the effect of the same concentrations on ZAP-stimulated Luci-DCL of peripheral blood neutrophils was examined. The Luci-DCL response to ZAP (10% v/v) was significantly attenuated at concentrations > 0.01% (data not shown). These findings were consistent with the effects of DTT reported previously (Wu *et al.*, 1997), so this method of mucus removal was not pursued.

A nylon gauze mesh (Nytex gauze, Nytex, UK, 60 μm pore size) was compared to cotton gauze for its ability to efficiently remove mucus from raw BALF without affecting cellular recovery or content. Absolute and differential cell counts of raw BALF from 11 randomly selected clinical cases were compared after filtration through one layer of either cotton gauze or sterile nylon mesh. There was no significant difference between absolute and differential cell counts compared to raw BALF with either filtration method but no visible mucus was present after filtration through nylon gauze (data not shown). Subsequently all BALF was filtered through sterile nylon gauze prior to further processing.

After filtration, BALF cells were pelleted (340 x *g*, 6 min) and the supernatant decanted and centrifuged further (1800 x *g*, 10 min) to remove any residual cellular or particulate debris and stored in aliquots at -70°C and -20°C until analysis.

BALF cells were washed twice (PBS w/o/0.1% BSA/10 mM HEPES, pH 7.4 and PBS /0.1% BSA/10 mM HEPES, pH 7.4; 235 x *g*, 6 min) prior to resuspension at 12.5 x 10^6/ml in PBS for chemiluminescence and at 0.5 x 10^6/ml in PBS /0.1% BSA, pH 7.4 for preparation of cytospins for immunocytochemical staining.

2.8.2 BRONCHOALVEOLAR LAVAGE FLUID CYTOLOGY

Differential cell counts were determined by counting 500 cells on duplicate Leishman's-stained cytospins prepared by centrifuging 100 µl raw BALF (300 x *g*, 3 min). Absolute BALF cell counts were determined for each cell type by multiplying the cell ratio (%) by the total cell count/100. Duplicate Diff Quik-stained cytospins were prepared for enumeration of apoptotic neutrophils (See Section 6.2.5) as morphologic identification of these cells was clearer than on Leishman's-stained slides. Cells were identified using established morphological criteria for equine BALF (McGorum and Dixon, 1994; Moore and Cox, 1996; Freeman and Roszel, 1997). Neutrophils, lymphocytes, macrophages, eosinophils, mast cells, basophiloid cells (McGorum and Dixon, 1994) and epithelial cells were counted separately.

2.8.3 ISOLATION OF NEUTROPHILS FROM BALF

2.8.3.1 Density gradient centrifugation

Although other workers (Grunig *et al.*, 1990; Tremblay *et al.*, 1993) have failed to purify neutrophils from equine BALF with sufficient purity and recovery for meaningful *in vitro* studies, this has been achieved by density gradient centrifugation in other species (Cerasoli *et al.*, 1988; Koh *et al.*, 1993; Brown *et al.*, 1995; Delclaux *et al.*, 1997).

Utilising the same protocol described for the isolation of peripheral blood neutrophils (Section 2.1), a broad range of cell densities was apparent in the post-challenge BALF samples, as determined from differential counts of cells harvested from both density interfaces and the cell pellet. Although neutrophils were enriched at the 42%/51% interface (4.4% neutrophils to 23.2% and 16.6% to 63.5% in 2 representative experiments), significant numbers of both lymphocytes and macrophages were also present. It appeared that, not only was there a broad range of cell densities in post-challenge BALF, but that these densities varied between horses and with the duration of challenge and timing of the BAL. Different plasma/Percoll density gradients were employed in an attempt to accommodate this (combinations of plasma/Percoll dilutions ranging from 30-48% (2% steps) in the upper layer and 50-

60% in the lower layer) but these only ever resulted in a modest enrichment of the neutrophil dominant band.

Two multi-step Percoll gradients reported to successfully resolve human and sheep BALF neutrophils (Cerasoli *et al.*, 1988) and human blood eosinophils (Stern *et al.*, 1992) respectively, and a commercial leucocyte-resolving medium (Histopaque 1077) were also employed with similarly unsatisfactory results.

Continuous isotonic Percoll gradients were generated to provide a more versatile method of resolving cells of very similar density. These were generated by centrifugation (58,000 x *g*, 30 min, 4°C) of 70% isotonic Percoll/PBS in a 34° fixed angle rotor centrifuge (Sorval RC-5B, Sorval UK Ltd., Stevenage, UK) against a reference tube containing marker beads (Pharmacia, Uppsala, Sweden) of known density (1033, 1049, 1062, 1075, 1087, 1098, 1122 and 1141 SG), (Figure 2.7). The performance of the gradients was also assessed using mixed peripheral blood leucocytes. Gradients were centrifuged (400 x *g*, 10 min, room temperature) and the purity of cells harvested from the visible isopycnic bands assessed. The mixed blood leucocytes were resolved into three bands (Figure 2.7) composed of 99.6 ± 0.05% (n = 3) mononuclear cells; 99.6 ± 0.4% neutrophils and erythrocytes/eosinophils respectively. BALF cells did not resolve uniformly, typically lying in two broad bands with many cells interspersed between these two bands (Figure 2.7). Differential counts of the cells harvested from the bands showed them to be very mixed populations (see legend to Figure 2.7). This confirmed that after challenge there was considerable overlap in the densities of the different cell types collected from the airspaces by BAL and it was not possible to resolve them into sufficiently pure populations based on their biophysical properties alone.

2.8.3.2 Immunological methods

When methods for cell purification based on biophysical properties have proved impractical, positive or negative selection of phenotypically homogenous cell populations with immunomagnetic beads has been used (Stern *et al.*, 1992). Although a commercial supplier (StemSep, Metachem Diagnostics Ltd., Northampton, UK) offered to attempt to establish and supply a panel of immunomagnetic reagents for negative selection of neutrophils from equine BALF,

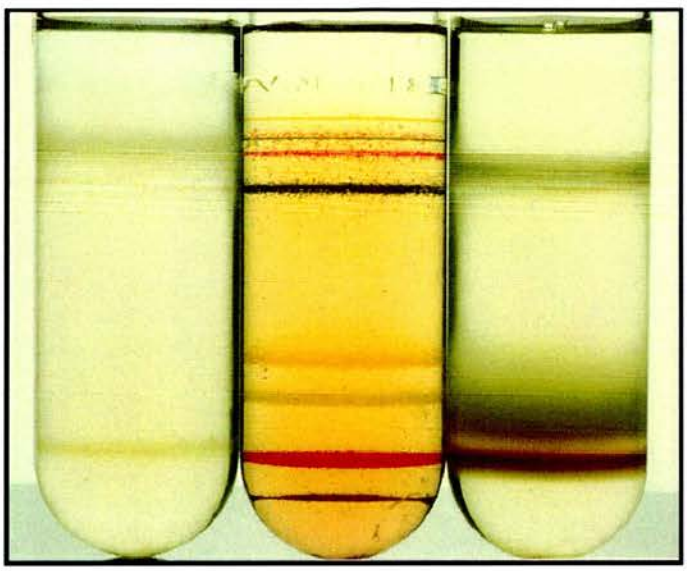


Figure 2.7: Separation of mixed peripheral blood and BALF leucocytes on continuous Percoll gradients

Left gradient: BALF cells; upper band - neutrophils 57.5%, lymphocytes 32.0%, macrophages 10.1%; lower band - neutrophils 50.9%, lymphocytes 20.3%, macrophages 28.7%, eosinophils 0.1%.

Middle gradient: Density marker beads

Right gradient: Mixed blood leucocytes; upper band - neutrophils 0.5%, lymphocytes 65.3%, monocytes 34.2%; middle band - neutrophils 100%; lower band - erythrocytes and eosinophils.

lack of funds precluded pursuing this method further.

2.8.3.3 Flow cytometric methods

Recently, a method for the flow cytometric determination of oxidative burst in BALF leucocytes collected from normal horses has been described (Raidal *et al.*, 1998a). However, previous work in this laboratory had indicated that it was not possible to successfully resolve individual populations of BALF leucocytes after hay/straw challenge based upon their forward and side light scatter profiles alone (McGorum *et al.*, 1993b). This would prevent accurate gate analysis of a pure neutrophil population

2.8.3.4 Measurement of neutrophil specific chemiluminescence

One of the goals of isolating neutrophils from equine BALF was to assess changes in their oxidative activity following recruitment to the lung. The mechanisms underlying the CL response of phagocytes in the presence of different luminigenic probes suggested that it might be possible to differentiate the CL signal generated by neutrophils from that generated by other BALF phagocytes. Lum-DCL from phagocytes is almost entirely dependent upon the haemoprotein myeloperoxidase (Aniansson *et al.*, 1984; Allen, 1986; Benbarek *et al.*, 1996) found in the azurophilic granules of neutrophils and the lysosomes of monocytes (Klebanoff, 1991), whereas Luci-DCL is independent of myeloperoxidase and associated with superoxide anion generation (Aniansson *et al.*, 1984; Allen, 1986; Benbarek *et al.*, 1996). Moreover, as monocytes mature or migrate into tissues and transform into macrophages *in vivo*, their myeloperoxidase is lost (Nichols *et al.*, 1971; Borregaard *et al.*, 1993), suggesting that they would produce little if any Lum-DCL. It has been confirmed for human BALF cells, from CL studies of alveolar macrophages mixed with peripheral blood neutrophils *in vitro* (Ward *et al.*, 1990) and from BALF harvested from patients with various inflammatory lung diseases (Williams and Cole, 1981; Ward *et al.*, 1990; Dalhoff *et al.*, 1994) that Lum-DCL almost exclusively reflects neutrophil respiratory burst activity. We tested this hypothesis for equine BALF cells. BALF from four horses, processed as described in Section 2.9.1, was supplemented with allogenic peripheral blood neutrophils to produce samples containing 0-90%

blood neutrophils (10% steps) whilst maintaining the cell concentration. Basal (buffer only) and ZAP-stimulated Luci- and Lum-DCL were measured. Data from the four animals were pooled and linear regression coefficients were calculated by least squares analysis for the relationships between Luci- and Lum-DCL and neutrophil and alveolar macrophage counts. ZAP-stimulated Lum-DCL increased linearly with increasing PMN percentage and this correlation was highly significant (Figure 2.8a). No other significant positive linear relationships were recognized. This indicated that ZAP-stimulated Lum-DCL was almost exclusively generated by PMN and might be used as an index of BALF PMN oxidative activity *ex vivo*.
To confirm that this conclusion remained valid with a mixed population of BALF cells *in vivo*, the relationships between PMN counts and PBS-, fMLP-, ZAP- and PMA-stimulated Luci- and Lum-DCL of BALF cells collected during the hay/straw challenge experiments were analysed retrospectively (Spearman Rank correlation coefficients). Data from all time points were pooled for this analysis. PBS-, fMLP-, ZAP- and PMA-stimulated Lum-DCL were all strongly correlated ($r = 0.70$, 0.67, 0.76 and 0.78, respectively, $p<0.0001$ for all measurements) with the BALF neutrophil percentage. The correlation between ZAP-stimulated Lum-DCL and BALF neutrophil percentage is shown in Figure 2.8b. The weaker correlation between the Lum-DCL of post-challenge BALF cells and the proportion of neutrophils compared to the experiments using peripheral blood neutrophils shown in Figure 2.8a may have been due to differences in the priming status and hence respiratory burst activity of neutrophils recruited to the lung after challenge (discussed in detail in Chapter 6). PMA- and fMLP-stimulated Luci-DCL were less strongly correlated ($r = 0.489$ and 0.48, respectively; $p<0.01$) with the BALF neutrophil percentage. These findings suggested that it was appropriate to quantify the effect of hay/straw challenge on BALF phagocyte oxidative activity by CL and that Lum-DCL could be used as an index of BALF neutrophil respiratory burst activity.

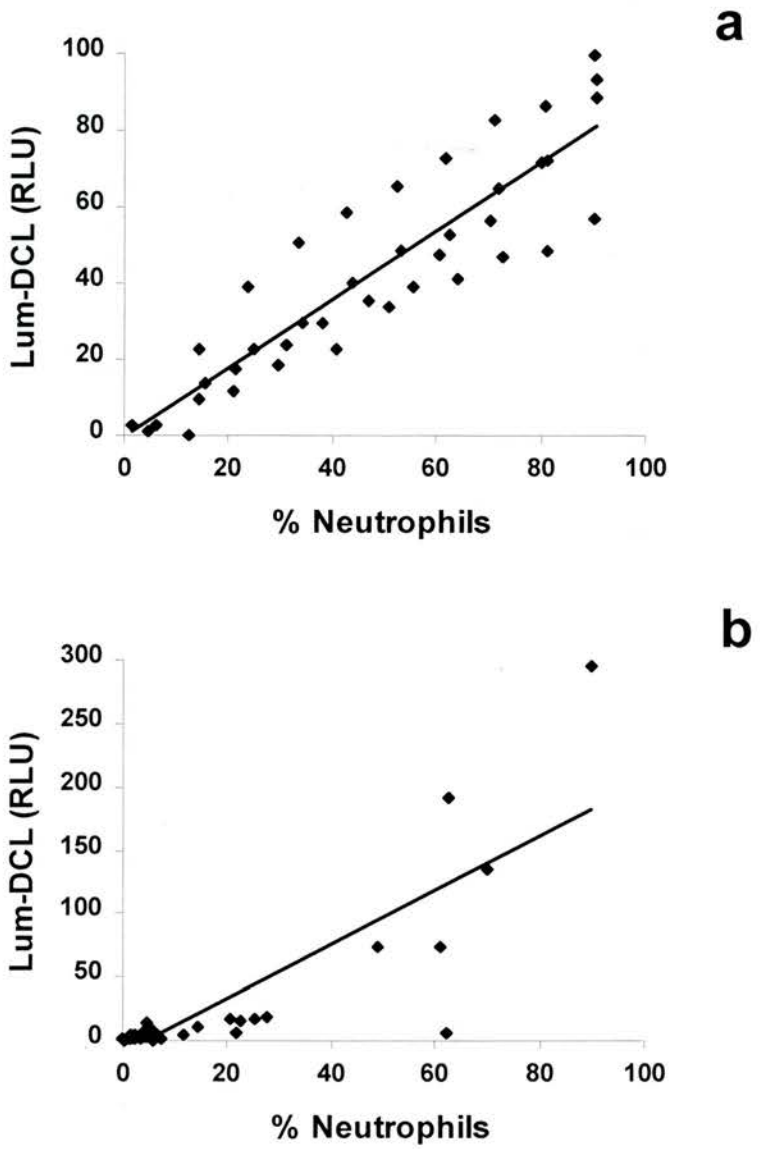


Figure 2.8a,b: Lum-DCL of equine BALF cells is neutrophil-dependent

a: BALF cells (processed as described in Section 2.9.1) were supplemented with allogenic peripheral blood neutrophils to produce samples containing 0-90% neutrophils. ZAP-stimulated Lum-DCL (measured over 90 min) increased linearly with the percentage of neutrophils (linear regression co-efficient calculated by least squares analysis, $r^2 = 0.81$, $p<0.0001$). Pooled data from four experiments.

b: ZAP-stimulated Lum-DCL of BALF cells harvested in challenge experiments (processed as described in Section 2.9.1). Lum-DCL and percentage PMN in BALF were closely correlated (Spearman Rank correlation co-efficient $r = 0.76$, $p<0.0001$). Pooled data from 6 time points ($n = 6$).

2.8.4 MEASUREMENT OF RESPIRATORY BURST ACTIVITY IN BALF CELLS BY CHEMILUMINESCENCE

Lucigenin- and Lum-DCL of BALF cells and isolated peripheral blood neutrophils were measured in parallel in response to PBS, fMLP (1 μM), ZAP (10% v/v) and PMA (100 ng/ml) at each pre- and post-challenge time point. In these experiments, following isolation, blood neutrophils were washed in an identical manner to that described for BALF cells (Section 2.9.1).

PBS- and fMLP-stimulated CL was recorded over a 5 min time course and PBS-, ZAP- and PMA-stimulated CL was recorded over a 90 min time course. Concentrations of fMLP, ZAP and PMA were selected from the concentration response curves generated during earlier *in vitro* studies (Sections 3.2.1; ZAP and PMA and 3.2.3.2; fMLP).

To ensure that any changes in BALF neutrophil function were not caused by the trauma of endoscopic BALF collection *per se*, *in vitro* sham lavage experiments were performed using peripheral blood neutrophils harvested from horses (n = 3) in remission. Two aliquots of neutrophils (100 x 10^6) were resuspended at 1 x 10^6/ml in 0.9% NaCl at 37°C. One aliquot was aspirated through the endoscope by hand using a 60 ml syringe (BAL sample) and the other was maintained on the bench at room temperature (Control sample). Half the cells in each aliquot were prepared (see Section 2.9.1) immediately (t = 0) and PBS- and fMLP-stimulated Luci- and Lum-DCL were measured as described above. The remaining neutrophils were allowed to stand for 90 min (the maximum recorded delay between BAL and measurement of BALF cell CL) prior to preparation and measurement of CL (t = 90). There was no significant difference in either the Luci- or Lum-DCL response of the neutrophils that had undergone the sham BAL procedure compared to control cells at either time point (Luci-DCL data [RLU]: t = 0; Control, PBS 0.08±0.02, BAL, PBS 0.02±0.02, Control, fMLP 0.24±0.09, BAL, fMLP 0.49±0.04. t = 90; Control, PBS 0.04±0.01, BAL, PBS 0.1±0.02, Control, fMLP 0.28±0.07, BAL, fMLP 0.40±0.09, n = 3, $p>0.05$).

2.8.5 MEASUREMENT OF EQUINE NEUTROPHIL ELASTASE TYPE 2A (ENE 2A) IN BALF SUPERNATANT BY ELISA

Purification of ENE 2A, production and characterisation of an ovine polyclonal antibody to ENE 2A and the development of the specific sandwich ELISA for ENE 2A was performed by Dr M. P. Dagleish, Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies (Dagleish, 1999a).

Micro-ELISA plate wells (Dynex Technologies Ltd., Billingshurst, UK) were coated with 50 µl of sheep anti-ENE 2A (1.5 µg/ml) in 0.1 M carbonate/bicarbonate buffer. Following overnight incubation at 4°C, plates were washed 6 times with 0.9% NaCl containing 0.05% Tween 20 (0.9% NaCl-T20). Samples (in duplicate) and standards (in triplicate) were diluted in 5% dried skimmed milk in PBS (MPBS) containing 0.05% Tween 20; 50 µl of each standard or sample were added per well. Purified ENE 2A was used to generate a standard curve over the range 0.5 to 4.0 ng/ml. After addition of samples and standards, plates were incubated at room temperature (21°C) for 1 h followed by washing 6 times in 0.9% NaCl-T20. Fifty microlitres of affinity purified sheep anti-ENE 2A conjugated to horseradish peroxidase (HRPO) (1 µg/ml in 5% MPBS containing 0.05% Tween 20) was added per well and the plates were incubated for a further hour at room temperature followed by a final 6 washes in 0.9% NaCl-T20. To initiate colour development 50 µl of 3,3',5,5'-tetramethylbenzidine (TMB - Dynex Technologies Ltd., Billingshurst, UK) was added per well as a soluble substrate for the HRPO enzyme and the reaction terminated by addition of 25 µl of 0.18 M H_2SO_4. The colour intensity was measured at 490 nm on a MR7000 ELISA plate reader (Dynex Technologies Ltd., Billingshurst, UK). ENE 2A is recognized by ELISA whether in the free or complexed state (Dagleish, 1999a).

To ensure that ENE release from neutrophils was not induced by the trauma of endoscopic BALF collection *per se*, *in vitro* sham lavage experiments (n = 3) were performed as described in Section 2.9.4 above. Cell-free supernatants were prepared as described in Section 2.8.1 both immediately after aspiration and 90 min later, prior to measurement of ENE 2A by ELISA. Only small amounts (< 10 ng/ml) of ENE

2A were detectable in the supernatants from both sham BAL and control cells at both time points and these were not significantly different (data not shown).

2.8.6 MEASUREMENT OF EQUINE NEUTROPHIL ELASTASE ACTIVITY IN BALF SUPERNATANT

Elastase activity in BALF supernatant was assayed using the specific neutrophil elastase substrate N-methoxysuccinyl-ALA-ALA-PRO-VAL p-nitroanilide (Nakajima *et al.*, 1979).

The substrate was dissolved in dimethyl sulphoxide (10 mg/ml) and stored at 4°C until use. Standards (ENE 2A) were diluted in buffer (50 mM Tris, 0.5 M NaCl, 0.1% triton X100, pH 8.0) and 50 µl of each standard or sample were added, in triplicate, to a 96-well plate. Supernatant samples from all horses at all time points were measured on a single plate. Purified ENE 2A was used as a standard over the range 0.16 - 20 ng/50µl. Following the addition of 50 µl substrate (0.1 ng/ml in buffer) the change in absorbance produced by cleavage of the chromogenic substrate was measured at 405 nm in a MR7000 ELISA plate reader (Dynex Technologies Ltd., Billingshurst, UK). Absorbance was measured 1 min after addition of the substrate and then at regular intervals until the optical density values of the 20 ng ENE 2A/50 µl standard wells reached a plateau (typically 90 min). A standard curve was generated from the change in absorbance (absorbance at plateau - absorbance at 1 min) of the ENE 2A standards from which the concentration of elastase in the BALF samples could be computed. The long-term stability of N-methoxysuccinyl-ALA-ALA-PRO-VAL p-nitroanilide facilitates a very sensitive assay because, providing the concentration of the chromogenic substrate is non-limiting, the change in absorbance resulting from cleavage by even very low concentrations of elastase remains linear for at least 12 h (Delclaux *et al.*, 1997). In parallel experiments, absorbance was repeatedly measured over 18 h, in an attempt to detect very low concentrations of ENE. The change in absorbance with the 0.16 ng ENE 2A/50 µl standard remained linear over this period (data not shown).

2.8.7 IMMUNOCYTOCHEMICAL LOCALISATION OF ENE 2A IN BALF CELLS

For immunocytochemical evaluation, cytospins of peripheral blood leucocytes and BALF cells were prepared on TESPA-coated slides and fixed in freshly prepared 4% paraformaldehyde in PBS (45 min, 45°C). Thereafter the slides were transferred to 70% ethanol and stored at 4°C. Cytospin preparations were then further dehydrated in 95% ethanol and endogenous tissue peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 20 min followed by washing in running tap water for 5 min. Slides were placed in an humidified chamber and non-specific binding of the rabbit anti-sheep antibody eliminated by incubating cells in 5% normal rabbit serum in PBS (5% NRS/PBS) for 30 min, followed by incubation with the sheep anti-ENE 2A antibody (2.5 μg/ml in 5% NRS/PBS) for 90 min. Slides were washed three times (5 min each) in PBS, prior to the addition of a rabbit anti-sheep antibody conjugated to horseradish peroxidase (Dako Ltd., High Wycombe, UK) diluted 1/400 in 5% NRS/PBS for 60 min. Three washes in PBS (5 min each) preceded staining with a 3-amino-9-ethylcarbazole (AEC) peroxidase substrate kit (Vector Laboratories, Peterborough, UK) used as per the manufacturer's instructions. Following the appearance of grossly visible red colouration (10-12 min) the slides were washed in running tap water for 2 min followed by counterstaining in Mayer's Haematoxylin and blueing with Scott's Tap Water Replacement. Slides were initially mounted in Crystal/Mount (Biomeda Corp., Foster City, USA) followed by DPX mounting medium. Preparation of cytospins of mixed peripheral blood leucocyte populations indicated that the antibody identified exclusively neutrophils. Negative control preparations were treated identically except that the sheep anti-ENE antibody was substituted with an identical concentration of normal sheep IgG (2.5 μg/ml in 5% NRS/PBS).

2.8.8 IMMUNOCYTOCHEMICAL LOCALISATION OF APOPTOTIC CHROMATIN IN BALF CELLS BY THE TUNEL METHOD

To confirm that the densely staining inclusions recognized in alveolar macrophages on Diff Quik-stained cytospins of BALF represented apoptotic nuclear chromatin, additional cytospins were prepared from BALF cells collected 24 h after challenge. These were fixed and stained for terminal deoxynucleotide transferase mediated UTP nick end-labelling (TUNEL) as described in Section 2.6.6.

2.8.9 ASSESSMENT OF ULTRASTRUCTURAL MORPHOLOGY OF BALF CELLS

To aid the identification of apoptotic neutrophils present both free within BALF and within phagocytic alveolar macrophages, BALF cells were examined ultrastructurally by TEM. BALF was collected from horses 24 h after challenge and 40 x 10^6 cells were washed, fixed, prepared, examined and imaged as described in Section 2.3.2.1.

2.8.10 MEASUREMENT OF IL-8 AND TNF-α IN EQUINE BALF

Equine IL-8 was measured in the BALF supernatant by Dr M. Francini, Institute for Veterinary Physiology, University of Zurich, Switzerland, who has previously cloned and expressed erIL-8 and prepared a polyclonal rabbit anti-erIL-8 antiserum (unpublished data). Triplicate samples of BALF supernatant and erIL-8 standards (100 pg - 100 ng/ml) were blotted onto a nitrocellulose membrane and incubated (30 min, room temperature) in assay buffer (PBS/1% Tween/1% BSA). The membrane was washed in PBS, incubated in assay buffer containing the polyclonal rabbit anti-erIL-8 antiserum (90 min, room temperature) and washed a further three times in assay buffer prior to incubation with an alkaline phosphatase-conjugated mouse anti-rabbit monoclonal antibody (45 min, room temperature). After further washing in PBS, the nitrocellulose was immersed in substrate solution until a dark spot was readily recognizable at the site of the 100 ng/ml erIL-8. The membrane was washed

in distilled water, air dried and the test and standard reactions digitised and analysed by densitometry (Gel-Doc 2000, Bio-Rad, Hercules, USA) to generate a standard curve and permit determination of the concentration of IL-8 in the test samples. Additional attempts were made to measure equine TNF-α and IL-8 using human and ovine cytokine ELISA systems by Mr J. Deighton, Department of Medicine, Cambridge University School of Clinical Medicine. Three methods were utilised: (i) a human assay system using monoclonal anti-human TNF-α and IL-8 capture antibodies, with detection by a polyclonal anti-species conjugated antibody; (ii) a second human ELISA system, using plates coated with a polyclonal anti-human IL-8 and detection with a biotinylated polyclonal anti-human IL-8 antibody and extra-avidin conjugated alkaline phosphatase, and (iii) an ovine cytokine ELISA system using a monoclonal anti-sheep IL-8 coating antibody with detection using a secondary polyclonal conjugated anti-sheep IL-8 antibody. In all systems standard curves were generated using the appropriate species recombinant cytokine.

2.9 STATISTICAL ANALYSES OF DATA

Data from the *in vitro* studies of neutrophil function were expressed as mean ± SEM values of (n) number of independent experiments. Data were analysed using ANOVA followed by the Student-Newman-Keuls post-test or by the student's paired t-test. The effect of LPS priming on [^{3}H]fMLP binding to equine neutrophils was analysed using a paired one-tailed student's t-test. EC_{50} values were obtained by analysis using Kaleidagraph (Macintosh) or GraphPad Prism (GraphPad Software Inc.) software.

Data collected from the *in vivo* challenge studies and *ex vivo* neutrophil function experiments were not normally distributed and were expressed as median and (range) values for n animals. Consequently, paired and unpaired analyses of these data were performed using the Wilcoxon Sign Rank and Mann Whitney tests, respectively. Correlations were examined using the Spearman Rank Sign test and where one variable was controlled, linear regression coefficients were calculated by least squares analysis for the relationship to a measured variable. Multiple groups (e.g.

Sham BAL data from different lung segments) were compared using the Kruskal-Wallis test. Within group analyses of data collected from the *in* and *ex vivo* hay/straw challenge studies were performed by comparison of data from post-challenge time points with that collected at baseline. Analyses were performed using either Minitab (Minitab Inc., Pennsylvania, USA) or GraphPad Prism (GraphPad Software Inc.). Results were considered to be significant at the 5% level.

2.10 MATERIALS

2.10.1 REAGENTS AND LABORATORY EQUIPMENT

Bakers' yeast (*Saccharomyces cerevisae*), BSA, DNase 1, Dextran T500, dithriothreitol, fMLP, glutaraldehyde, HEPES buffer, Histopaque 1077, LPS from *Escherichia coli* serotype 0111:B4, lucigenin, luminol, Mayer's Haematoxylin, methylene blue, normal sheep IgG, normal rabbit serum, N-methoxysuccinyl-ALA-ALA-PRO-VAL p-nitroanilide, penicillin/streptomycin, PMA, PBS, PBS w/o, rabbit anti-sheep HRPO conjugated antibody, RNase A, TESPA, Trizma base, triton X-100, Tween 20, trypan blue and zymosan were purchased from Sigma (Poole, Dorset, UK). Iscove's modified Dulbecco's medium, Dulbecco's Modified Eagle Minimal Essential Medium and HBSS were purchased from Gibco Life Technologies (Paisley, UK). 0.9% NaCl was purchased from Ivex Pharmaceuticals (Larne, N. Ireland). All tissue culture reagents were certified as endotoxin-free by the manufacturers. Percoll was obtained from Pharmacia (Uppsala, Sweden). Laboratory plasticware was purchased from Becton Dickinson Labware (Oxford, UK) or Alpha Laboratories (Eastleigh, UK) unless otherwise stated. Round-bottomed 2 ml Eppendorf tubes were purchased from Eppendorf (Hamburg, Germany). Cotton gauze (Propax) was purchased from Smith & Nephew (Brierfield, UK). 3.8% sodium citrate was purchased from Phoenix Pharmaceuticals Ltd (Gloucester, UK). Silicone oil F-50 was obtained from Croylek Ltd, (Surrey, UK). PAF and LTB_4 were purchased from Novabiochem (Nottingham, UK). Rough mutant *Salmonella typhimurium* Ra 60 was a gift from Dr I. Poxton, Department of Microbiology, Medical School, University of Edinburgh. ErTNFα was a gift from

Dr M. Barton, Department of Large Animal Medicine, University of Georgia, USA and the HL801 hybridoma cell line producing erTNFα neutralising antibody was a gift from Dr R. MacKay, Department of Large Animal Medicine, University of Florida, USA. Annexin V - FITC and Annexin V Buffer were purchased from Biowhittaker (Wokingham, UK). HrTNFα and hrGM-CSF and hrIL-8 were purchased from Genzyme (Cambridge, MA). The hrTNFα neutralising antibody (MAB 210) and mouse isotype control (IgG_1) antibody (MAB 002) were purchased from R & D Systems (Abingdon, UK). Canine anti-sheep red blood cell antibody was purchased from VMRD, Inc, (Pullman, USA). [^{3}H]fMLP (specific activity 1.48-3.219 TBq [40-87 Ci/mmol]) was purchased from NEN Life Science Products (Stevenage, UK). Scintillation fluid (Flo-Scint IV) was purchased from Packard Biosciences B.V. (Groningen, Netherlands). Diff Quik was purchased from B M Brown Ltd (Reading, UK). Leishman's stain and DPX mounting medium were purchased BDH (Loughborough, UK). All other chemicals were of molecular or reagent grade and were obtained from Sigma (Poole, UK) or BDH (Loughborough, UK).

2.10.2 PREPARATION AND STORAGE OF REAGENTS

Stock LPS (1 mg/ml in PBS) was stored at -20°C and once thawed was disaggregated in a sonicating waterbath for 10 min. Stock PAF (10 mM in ethanol), PMA (dissolved in DMSO, diluted to 1 mg/ml in PBS), fMLP (dissolved in DMSO, diluted to 1 mM in PBS) and LTB_4 (1.49×10^{-4} M in ethanol) were stored at -20°C. Stock hrTNFα (10 μg/ml in PBS), erTNFα (12.5 ng/ml in PBS) and hrIL-8 (10 μg/ml in PBS) were stored at -80°C. All antibodies were stored at -20°C. All other reagents were stored as per the manufacturers' instructions.

Autologous heat-inactivated serum was prepared by the addition of 220 μl of 10 mM $CaCl_2$ to10 ml of platelet-rich plasma, incubating for 90 min at 37°C in an humidified 5% CO_2 atmosphere, then heating at 56°C for 30 min.

Zymosan-activated plasma (ZAP) and serum (ZAS) were used as a source of the complement fragment C5a and were produced by adding 5 mg/ml zymosan to PPP or

serum followed by sonication and incubation at 37°C for 60 min. The suspension was centrifuged (1400 x *g*, 15 min) and the supernatant removed and stored in 100 µl aliquots at -20°C. ZAP and ZAS were used in all experiments at a final concentration of 10% (v/v). The same batches of ZAP and ZAS were used for all *in vitro* studies and fresh batches were prepared for use in the *ex vivo* hay/straw challenge studies.

Heat killed baker's yeast (5 g /100 ml PBS, boiled, 30 min) were washed twice in PBS, disaggregated by aspiration (twice) through a 29 G needle, washed again and opsonised by incubation with 10% pooled horse serum/PBS (50 x 10^6 particles/ml) at 37°C for 30 min (Johannisson *et al.*, 1995). The yeast were again washed twice and disaggregated, then resuspended at appropriate concentrations in PBS and stored in aliquots at -20°C. Control particles were prepared in an identical manner except they were incubated with PBS alone.

Opsonised ovine erythrocytes (OsRBC) were prepared freshly as required. Ten ml of ovine blood, anticoagulated with 3.8% sodium citrate, was centrifuged (800 x *g*, 10 min). Two ml of the RBC pellet was washed three times in PBS, diluted in PBS to a haematocrit of 1% and incubated (37°C, 30 min) with a sub-aggregating concentration (1/40 from stock) of canine anti-sheep red blood cell antibody. Cells were washed twice then resuspended at appropriate concentrations in PBS (CL studies) or Iscove's modified Dulbecco's medium (apoptosis studies). Control cells (sRBC) were prepared in an identical manner except they were incubated with PBS alone.

HL801 hybridoma cells (200,000 - 300,000/ml) were cultured in Dulbecco's Modified Eagle Minimal Essential Medium (high glucose with L-glutamine, without sodium pyruvate) supplemented with 10% horse serum and 100 U/ml penicillin/streptomycin at 37°C in an humidified 5% CO_2 atmosphere. For *in vitro* antibody production, cells were cultured in Dulbecco's Modified Eagle Minimal Essential Medium supplemented with ultra low IgG-containing foetal calf serum and 100 U/ml penicillin/streptomycin. Culture supernatant was harvested, centrifuged to remove cellular debris and stored at -20 °C.

Supernatant IgG1 concentration was measured using a commercial radial immunodiffusion kit (Nanorid, The Binding Site Ltd., Birmingham, UK).

Phosphate-Buffered Saline (PBS) for use in immunocytochemistry (Sections 2.6.6 and Section 2.8.7) was prepared by addition of 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 to 1 litre de-ionised, distilled water. pH was adjusted to 7.2-7.3.
The carbonate/bicarbonate buffer (0.1 M), ENE 2A ELISA coating buffer (Section 2.8.5) was prepared by addition of 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ was dissolved in 1 litre de-ionised, distilled water. pH was adjusted to 9.6 if necessary. The buffer was stored in 50 ml aliquots at -20°C.
Scott's Tap water replacement was prepared by addition of 7 g $NaHCO_3$ and 40 g $MgSO_4$ to 2 litres of tap water.
Scott's Tap water replacement and PBS were stored in sealed containers at room temperature.

CHAPTER 3

CHARACTERIZATION OF PRIMING AND ACTIVATION IN EQUINE PERIPHERAL BLOOD NEUTROPHILS *IN VITRO*

3.1 INTRODUCTION

The key role that neutrophils play in the vanguard of the acute cellular response to infection and injury necessitates tight co-ordination of the complex events that regulate both their recruitment to sites of inflammation and their ability to destroy invading micro-organisms (Haslett *et al.*, 1989b; Rossi and Hellewell, 1994; Bokoch, 1995; Downey *et al.*, 1995). Since these cells are recognized primarily as the linchpin of the innate immune response to invading micro-organisms, much attention has been focussed on the neutrophil's armoury of reactive oxygen and nitrogen species, proteolytic enzymes and antibacterial peptides (reviewed in Section 1.3). However, the "fallout" from this beneficial inflammatory response can, through the inappropriate or excessive secretion of histotoxic or pro-inflammatory substances by the neutrophil, contribute to host tissue damage. This sequence of events is increasingly being recognized and elucidated in the horse (reviewed in Section 1.6). As detailed in Section 1.4, the responsiveness of granulocytes to secretagogue stimuli is governed by whether these cells have been previously exposed to priming agents such as bacterial products (e.g. LPS and N-formylated peptides), cytokines (e.g. TNF-α, IL-8 and GM-CSF) and lipid-derived mediators (e.g. PAF and LTB_4) (Bochsler *et al.*, 1992; Condliffe *et al.*, 1998b). In many species, such agents sensitise the neutrophil to enhance certain functional responses, such as superoxide anion generation, adhesion, cell surface serine proteinase expression, release of proteolytic enzymes and the generation of lipid mediators, when subsequently exposed to secretagogue agonists (reviewed in Section 1.4.1). Priming also regulates the relative expression of surface adhesion molecules, most notably CD62-L (L-selectin) and the major β_2-integrin, CD18/CD11b (Condliffe *et al.*, 1996). Thus, the

augmentation of these effector functions following exposure to priming agents would seem to be a critical step to realise integrated and optimal neutrophil microbicidal activity. In addition, neutrophil priming is thought to be crucial to the initial recruitment of these cells to an inflamed site (Worthen *et al.*, 1987) and appears to be a prerequisite for neutrophil-mediated host tissue damage both *in vitro* (Smedly *et al.*, 1986) and *in vivo* (Worthen *et al.*, 1987; Williams *et al.*, 1993). It is important to note however, that priming appears to be a potentially reversible event [at least *in vitro*; (Kitchen *et al.*, 1996b)] and that the retention of primed cells in, for example, the pulmonary capillary bed does not necessarily equate with tissue damage (Ussov *et al.*, 1996).

To re-iterate, the key principal of the priming concept demands that exposure to a priming agent that potentiates a secretory response to a subsequent activating stimulus should not by itself elicit that response (Downey *et al.*, 1995; Condliffe *et al.*, 1998b). Indeed, pulling the trigger of the loaded "neutrophil" gun prior to selection of an appropriate target has the potential for disastrous consequences, not least if this occurs in intravascular neutrophils or cells still in the process of transiting the endothelium. *In vitro* studies suggest that unprimed, circulating neutrophils are entirely unresponsive to secretagogue stimuli such as fMLP (Elbim *et al.*, 1993; Pabst, 1994). This highlights the importance of using a neutrophil isolation method that does not prime cells when studying these events *in vitro* (Haslett *et al.*, 1985; Pabst, 1994).

Neutrophil priming was initially defined (Guthrie *et al.*, 1984) in studies examining the enhancement of secretagogue-induced oxidant generation by the NADPH oxidase; hence the focus on the respiratory burst in the characterization of this phenomenon in the horse. The chemiluminescence model used in these studies was specifically developed for its high sensitivity for detecting the kinetics of oxidant (especially superoxide anion) release from cells (Allen, 1986) and its flexibility in the study of the respiratory burst from a mixed population of cells (Ward *et al.*, 1990). Lucigenin only measures externally secreted oxidants (Dahlgren *et al.*, 1985) and the CL response is 99% inhibited by superoxide dismutase (Allen, 1986; Benbarek *et al.*, 1996) suggesting that such data is qualitatively similar to that

derived in the more traditional superoxide dismutase-inhibitable cytochrome C reduction assay (Guthrie *et al.*, 1984; Kitchen *et al.*, 1996b).

In preliminary experiments, a panel of potential neutrophil agonists was screened for their ability to stimulate the respiratory burst (Luci-DCL) in freshly isolated neutrophils.

The arachidonic acid metabolite, LTB_4 has been detected in inflammatory exudates in the horse (Higgins and Lees, 1984) and is a potent chemoattractant for equine neutrophils *in vitro* (Lees *et al.*, 1986; Watson *et al.*, 1987; Foster *et al.*, 1992). Leukotriene B_4 has also been shown to augment adherence (Marr *et al.*, 1999), the phagocytic and bactericidal capacity of equine neutrophils (Watson, 1988) and can stimulate recruitment of radiolabelled neutrophils to the lungs of normal horses (Marr *et al.*, 1998b). However, LTB_4 is known to be a weak secretagogue for human neutrophils (Rollins *et al.*, 1983; Snyderman and Uhing, 1992; Edwards, 1994b).

The synthetic tripeptide, fMLP, which mimics the effects of a number of bacterial cell wall-derived peptides, has been widely used as a model agonist for studying the response of mammalian neutrophils to bacterial infection, sepsis and tissue injury (Becker, 1987). This peptide has been detected in culture supernatants from a number of bacteria including *Escherichia coli* and in samples of dust associated with neutrophilic environmental lung disease in man (Siegel *et al.*, 1994). The large and consistent nature of the secretory and chemotactic response induced by fMLP in human neutrophils has also made it the agent of choice in studies exploring the mechanisms underlying receptor-mediated priming and activation (Becker, 1987). However, despite possessing a small number of seemingly identical high affinity fMLP receptors (Snyderman and Pike, 1980), equine neutrophils appear to display a very different pattern of responses compared to human neutrophils, to the extent that most recent reports have regarded the equine fMLP receptor as being of little functional significance (Camp and Leid, 1982; Sedgwick *et al.*, 1987; Scudamore *et al.*, 1993). This conclusion is supported by the lack of any inflammatory response when fMLP is injected intradermally in the horse, even at concentrations as high as 10 mM per 100 µl injection volume (McEwen and Lumsden, 1991). Despite this, a number of early *in vitro* studies have reported fMLP effects in equine neutrophils, including induction of chemotaxis [albeit only at very high concentrations (Zinkl and

Brown, 1982), and concentration-dependent lysosomal enzyme release (Snyderman and Pike, 1980). The latter observation suggested that superoxide anions might be produced under appropriate conditions since release of lysosomal enzymes and superoxide anions usually occur concurrently (Haslett *et al.*, 1985). Although two studies have stated that fMLP could induce superoxide anion generation in equine neutrophils (Snyderman and Goetzl, 1981; Bertram, 1985), no experimental data were presented to support these assertions. Hence, while specific species differences have been demonstrated in studies of inflammation in the horse (Slauson *et al.*, 1985; Bertram, 1985; Scudamore *et al.*, 1993; Lindberg *et al.*, 1998), the precise function and role of the fMLP receptor in equine neutrophils remains uncertain.

Interleukin-8 is a CXC chemokine, produced by many cell types including monocytes/macrophages, neutrophils and epithelial cells and is a potent and selective agonist of human neutrophils, stimulating shape change, adhesion, bioactive lipid synthesis, degranulation and the respiratory burst (Baggiolini *et al.*, 1994). There is, however, some controversy surrounding the ability of IL-8 to directly stimulate respiratory burst activity (Baggiolini *et al.*, 1992). For example, Elbim and colleagues (1994) demonstrated that IL-8 acts as a potent priming agent in human neutrophils, while others have suggested that this agent is, at best, a weak priming agent and that its more important functions are the regulation of neutrophil adhesion and migration (Roberts *et al.*, 1993). Interleukin-8 has been implicated in the pathogenesis of equine COPD (Franchini *et al.*, 1998) and hrIL-8 has been reported to augment adhesion of equine neutrophils to serum- and fibronectin-coated plastic in a CD18-dependent manner (Marr *et al.*, 1999). Francini and colleagues (1998) demonstrated an increase in the neutrophil chemotactic activity of BALF collected from COPD-susceptible horses when housed in dusty conditions. They related this to increased synthesis of IL-8 mRNA by alveolar macrophages stimulated *in vitro*.

Although not a physiological stimulus, the complex plant derived lipid, PMA has been widely used in neutrophil activation and signal transduction studies (Lehrer and Cohen, 1981; Lundqvist *et al.*, 1996), including a number of studies in equine neutrophils (Auer *et al.*, 1990; Bochsler *et al.*, 1990; Bochsler *et al.*, 1992; Benbarek *et al.*, 1996; Moore *et al.*, 1997; Marr *et al.*, 1997a; Marr *et al.*, 1999). PMA binds directly to protein kinase C (bypassing plasma membrane receptors), leading to its

sustained activation and redistribution with subsequent stimulation of neutrophil superoxide anion generation (Auer *et al.*, 1990; Bochsler *et al.*, 1992; Moore *et al.*, 1997; Marr *et al.*, 1997a), chemiluminescence (Benbarek *et al.*, 1996), adhesion (Marr *et al.*, 1999), aggregation and degranulation (Moore *et al.*, 1997) and β_2 integrin expression (Bochsler *et al.*, 1990).

Incubation of plasma or serum with the yeast cell wall component zymosan activates complement via the alternative pathway, generating the glycoprotein anaphylotoxins C3a and C5a (Edwards, 1994a). Only C5a is chemotactic for neutrophils and can also induce adhesion, degranulation and the respiratory burst (Edwards, 1994b). Although hrC5a can stimulate superoxide anion generation and augment adherence of equine neutrophils (Bochsler *et al.*, 1992; Foster *et al.*, 1997; Marr *et al.*, 1999), equine rC5a is not commercially available and therefore zymosan activated plasma (ZAP), as a biological source of C5a, has been used in the study of equine neutrophil activation (Sedgwick *et al.*, 1987; Slauson *et al.*, 1987).

A plethora of mammalian cell types are stimulated following exposure to lipopolysaccharide (LPS), a complex glycolipid component of gram-negative bacterial cell walls (Ulevitch and Tobias, 1995). Gram-negative bacterial sepsis and endotoxaemia following bacterial lysis are major factors implicated in the morbidity and mortality associated with acute lung injury and multi-organ failure in man and this endotoxin-associated lung injury is largely neutrophil dependent (Brigham and Meyrick, 1986; Welbourn and Young, 1992). Similarly, endotoxaemia and its subsequent complications appear to play a critical role in disease pathogenesis and mortality associated with gram-negative sepsis and gastro-intestinal mucosal injury in the horse (Hunt *et al.*, 1986; Morris, 1991; Moore *et al.*, 1995). Of particular interest to this study is the implication that LPS plays a central role in the response to inhaled organic toxic dust (Castranova *et al.*, 1996; Deetz *et al.*, 1997; Schenker *et al.*, 1998; Wohlford-Lenane *et al.*, 1999) and in the exacerbation of allergic asthma in humans (Michel *et al.*, 1996). Moreover, conventionally managed stables (horses bedded on straw and fed dry hay) contain total airborne LPS concentrations greater than those shown to cause bronchial hyperresponsiveness and pulmonary inflammation in normal human subjects (McGorum *et al.*, 1998). Indeed,

preliminary investigations in this laboratory suggest at least a complementary role for LPS in the pathogenesis of equine COPD (Pirie *et al.*, 1998).

Endotoxin has long been recognized as a potent priming agent of human neutrophils (Guthrie *et al.*, 1984; Haslett *et al.*, 1985; Pabst, 1994) and the mechanisms of cell interaction with LPS have undergone intense scrutiny (Wright, 1991; Ulevitch and Tobias, 1994; Ulevitch and Tobias, 1995). In view of the relative lack of information regarding the effects of endotoxin on equine neutrophils, the response of these cells to LPS and other human granulocyte priming agents was investigated. Bochsler and co-workers (1992) demonstrated that LPS binds avidly to equine neutrophils but is a weak stimulus of superoxide anion generation. Also, LPS alone failed to directly trigger a CL response in equine neutrophils (Benbarek *et al.*, 1997; Benbarek *et al.*, 1998). However, the data in these latter two studies were quite variable with neutrophils from several horses showing a clear concentration-dependent CL response to LPS. Smooth forms of LPS, as used in the studies reported herein, do not stimulate a CL response in human neutrophils, whereas rough forms, in which the variable length hydrophobic O-polysaccharide moiety is lost (Poxton, 1995), can directly activate the neutrophil respiratory burst (Kapp *et al.*, 1987).

The cytokine TNF-α is produced by a wide variety of cells involved in the acute inflammatory response, including macrophages, endothelial cells and neutrophils (Edwards, 1994b). TNF-α has been shown to play a key role in the response of the horse to endotoxaemia (MacKay, 1991; Cargile *et al.*, 1995). *In vitro*, TNF-α has been primarily recognized as a potent priming agent of human neutrophil function (McColl *et al.*, 1990; O'Flaherty *et al.*, 1991; Condliffe *et al.*, 1998b) and a stimulus for phagocytosis (Shalaby *et al.*, 1985; Klebanoff *et al.*, 1986) and adhesion to biological surfaces (Schleiffenbaum and Fehr, 1990) but alone it is a weak stimulus of superoxide anion generation (Kitchen *et al.*, 1996a). However, at high concentrations TNF-α may cause some direct respiratory burst activity of neutrophils in suspension (Klebanoff *et al.*, 1986; Kapp and Zeck-Kapp, 1990; Zeman *et al.*, 1996) and it is clearly able to stimulate superoxide generation in neutrophils that have been primed by other soluble agents or through adhesion (Schleiffenbaum and Fehr, 1990).

The acetylated phosphoglyceride lipid, PAF, is generated by the action of phospholipase A_2 on ether-linked phospholipids. Numerous cell types involved in the inflammatory response including neutrophils, platelets, monocytes/macrophages, mast cells and vascular endothelial cells (Chung, 1992) can release PAF. PAF has multiple effects on human neutrophils *in vitro*, stimulating cell polarisation and aggregation (Rossi *et al.*, 1993), chemotaxis (Shaw *et al.*, 1981), adhesion (Ingraham *et al.*, 1982), upregulation of surface adhesion molecules (Condliffe *et al.*, 1996) and it is a weak stimulus of the respiratory burst (Gay *et al.*, 1986). However, PAF also operates as a potent neutrophil priming agent for enhanced superoxide anion generation, aggregation and degranulation in response to fMLP (Vercellotti *et al.*, 1988; Gay, 1993; Kitchen *et al.*, 1996b). PAF has profound effects on cardiopulmonary function when administered to humans systemically (Chung, 1992). It is a potent mediator of equine neutrophil chemotaxis (Foster *et al.*, 1992) and adhesion (Foster *et al.*, 1997; Marr *et al.*, 1999) but a poor stimulus of superoxide anion generation (Marr *et al.*, 1997a). Intradermal injection of PAF produces dose-dependent increases in vascular permeability in the horse (Mills *et al.*, 1995) and intravenous administration stimulates rapid but reversible increases in heart rate, respiratory rate and pleural pressure with recruitment of neutrophils, eosinophils and platelets to the lungs (Fairbairn *et al.*, 1996).

Although this chapter focuses upon the neutrophil respiratory burst, other functional responses implicit in the neutrophil priming/activation paradigm have been studied as necessary. For example, exposure of neutrophils to uniform (i.e. non-gradient) concentrations of inflammatory mediators or chemoattractants *in vitro* stimulates many functional and biochemical changes, including cell polarization or shape change. This latter effect has a number of significant functional correlates; for example, microscopic scoring of neutrophil shape change correlates well with the chemotactic capacity of these cells measured using the Boyden chamber technique (Haston and Shields, 1985). More importantly, shape change correlates closely with the priming status of neutrophils measured by subsequent agonist-stimulated superoxide anion generation (Haslett *et al.*, 1985; Kitchen *et al.*, 1996b). Also priming-induced cytoskeletal alterations reduce neutrophil deformability, which, *in*

vivo increases their sequestration within the pulmonary capillary bed (Haslett *et al.*, 1987; Worthen *et al.*, 1989; Selby *et al.*, 1991). This study centres upon characterization of the CL response of the equine neutrophils to three secretagogue agonists PMA, ZAP and fMLP and the modulation of these responses following exposure to the well-substantiated human neutrophil priming agents LPS, TNF-α and PAF. This model of equine neutrophil priming was specifically developed with a view to its application to the *ex vivo* study of neutrophils harvested from horses susceptible to COPD. It was anticipated that this would provide an index of the functional status of equine neutrophils both in the circulating granulocyte pool and those recruited to the airspaces following exposure of these susceptible animals to a hay/straw challenge.

3.2 RESULTS

3.2.1 EQUINE NEUTROPHIL ACTIVATION: THE RESPIRATORY BURST

In preliminary experiments, a panel of potential neutrophil agonists was screened for their ability to stimulate Luci-DCL in freshly isolated cells. These included PMA, ZAP, human and equine rTNF-α, LPS, PAF, fMLP, LTB_4, and hrIL-8 as soluble stimuli and opsonised heat-killed yeast as a particulate stimulus.

Leukotriene B_4 (0.1 μM) stimulated a very small but significant response (control, 0.2 ± 0.03 RLU; LTB4, 0.3 ± 0.04 RLU; n = 11, $p < 0.0001$) suggesting that, as in human neutrophils, this agent is a relatively poor secretagogue.

Incubation of freshly isolated neutrophils with fMLP (1 μM) did not stimulate Luci-DCL compared to buffer treated control cells (control, 0.2 ± 0.04 RLU; fMLP, 0.3 ± 0.1 RLU; n = 17, $p > 0.05$) consistent with the work of Benbarek *et al.* (1996).

In this assay, hrIL-8 (1 μg/ml) did not stimulate a respiratory burst (control, 0.2 ± 0.03 RLU; hrIL-8, 0.2 ± 0.04 RLU; n = 11, $p > 0.05$).

3.2.1.1 Activation of the respiratory burst by PMA

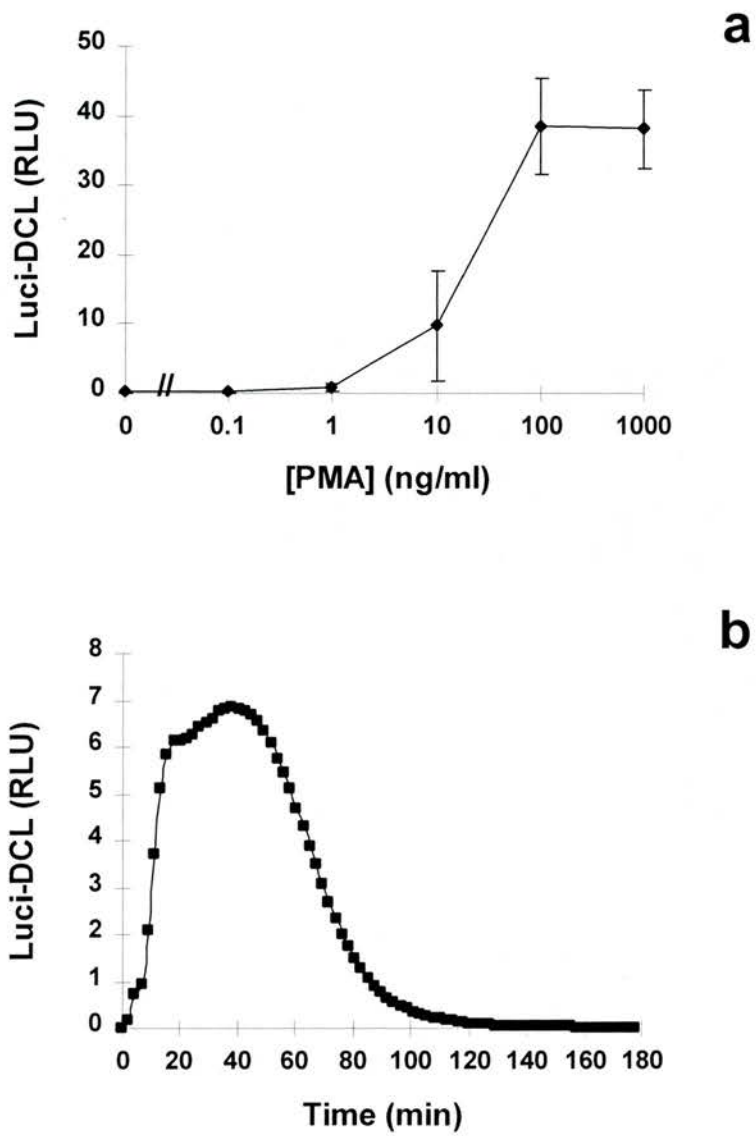
PMA stimulated concentration- and time-dependent Luci-DCL (Figure 3.1a,b) with an EC_{50} of 13.9 ± 2.5 ng/ml (n = 4), a maximal response to 100 ng/ml PMA with a nodal time of 34.6 ± 5.8 min. This is consistent with previously published data in equine cells [95% confidence interval of 0.4 - 9.9 ng/ml (Moore *et al.*, 1997) and an EC_{50} of 5 ng/ml (Marr, 1996)] and is very similar to the EC_{50} value of 6.5 ng/ml reported for human neutrophils (Lieberman *et al.*, 1996).

3.2.1.2 Activation of the respiratory burst by ZAP

ZAP stimulated a concentration-dependent respiratory burst (Figure 3.2a) over a time course similar to PMA but with a shorter nodal time (Figure 3.2b). While the duration of the CL response to ZAP did show some variation between donors (e.g. compare Figures 3.2b and 3.8), nodal times were very consistent. In all subsequent experiments, including the *ex vivo* challenge studies, CL was measured over 90 min using 10% v/v ZAP.

3.2.1.3 Activation of the respiratory burst by phagocytosis

Phagocytosis-stimulated Luci-DCL was measured following the addition of opsonised heat-killed yeast, or yeast incubated with PBS alone, at a ratio of 3 yeast particles per neutrophil. Phagocytosis was confirmed to have taken place by light microscopic examination of Diff-Quik stained cytospin preparations of cells harvested at the end of the assay (data not shown). Interaction with non-opsonised particles stimulated a significant respiratory burst but this was greatly enhanced ($p<0.01$, n=5) following opsonisation (Figure 3.3). The kinetics of the Luci-DCL response to opsonised yeast were also significantly faster (nodal time 23.3 ± 2.2 min, n = 5) than in response to non-opsonised yeast (nodal time 72.2 ± 10.3 min, $p<0.01$). This likely reflects an increase in both the rate and the number of particles ingested.



Figures 3.1a,b: Activation of equine neutrophil respiratory burst by PMA

a: Concentration-response curve for PMA-stimulated Luci-DCL in equine neutrophils. Values represent the mean ± SEM of 4 separate experiments, each performed in triplicate.

b: Time course of PMA-stimulated (100 ng/ml) Luci-DCL in equine neutrophils. The data points represent mean of triplicate determinations from a single experiment representative of ten. Nodal time: 38.6 min (mean: 34.6 ± 5.8 min).

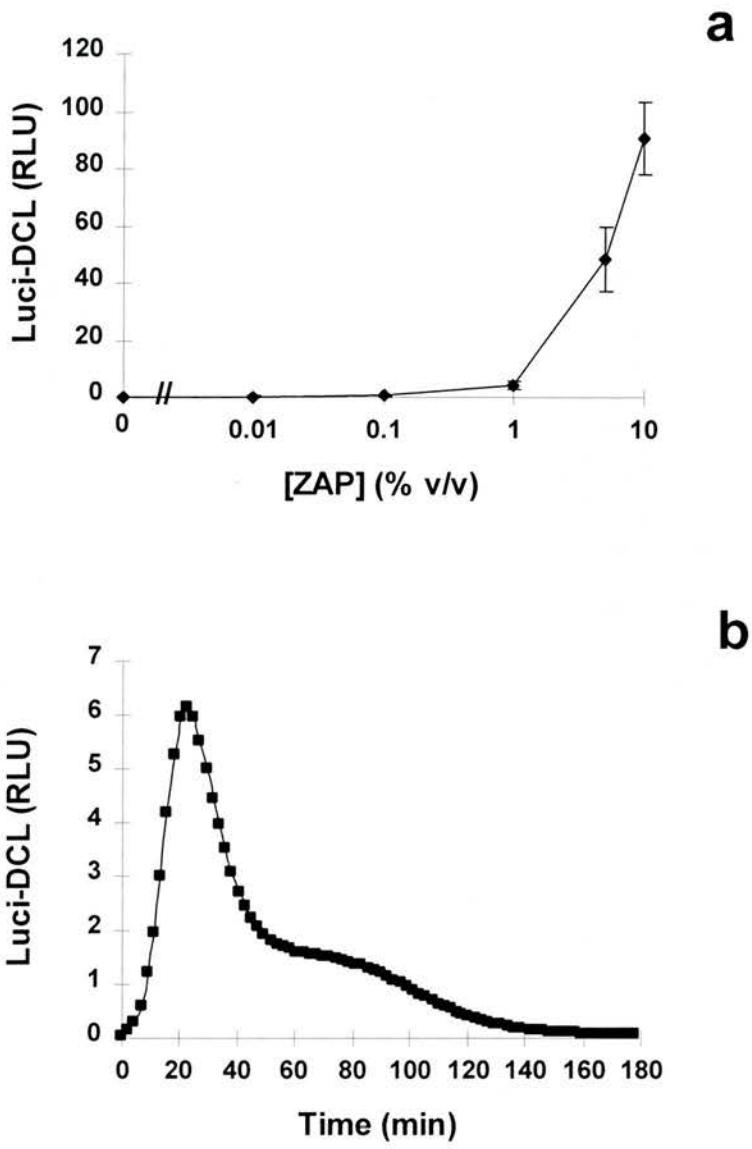


Figure 3.2a,b: Activation of equine neutrophil respiratory burst by ZAP

a: Concentration-response curve for ZAP-stimulated Luci-DCL. Values represent mean ± SEM of 3 separate experiments, each performed in triplicate.

b: Time course of ZAP-stimulated (10% v/v) Luci-DCL. Data points represent the mean of triplicate determinations from a single experiment representative of ten. Nodal time: 20.7 min (mean: 23.5 ± 1.05 min).

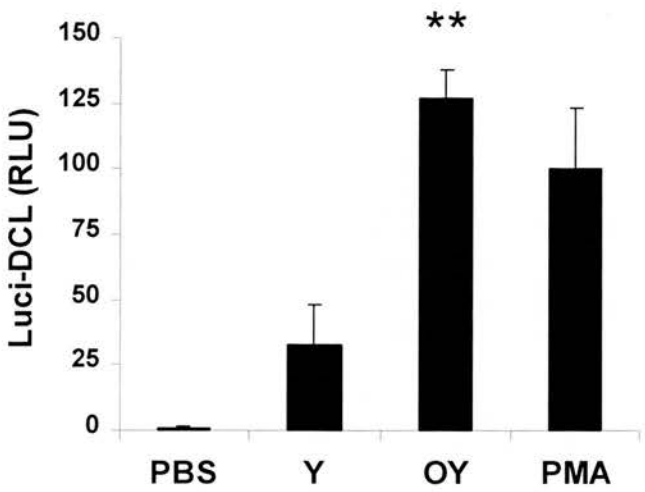


Figure 3.3: Phagocytosis-stimulated respiratory burst in equine neutrophils
Neutrophils were stimulated by addition of non-opsonised (Y) or opsonised yeast (OY) at a ratio of 3 yeast particles/cell or PMA (100 ng/ml) as a positive control. Luci-DCL was recorded over 90 min. Values represent mean ± SEM of integral data from 5 separate experiments (n = 3 for PMA), each performed in triplicate. (**; $p<0.01$, Y vs OY)

3.2.1.4 Comparison of Lucigenin- and Luminol-dependent chemiluminescence

As discussed in Section 3.1 above, luminigenic probe-dependent CL measured in the presence of lucigenin is generated predominantly by externally secreted superoxide anions (Benbarek *et al.*, 1996). Measurement of Luci-DCL permitted qualitative comparison of CL data with previously published studies of both human and equine neutrophils that utilised the superoxide dismutase-inhibitable cytochrome C reduction assay to quantify superoxide anion release. However, Lum-DCL [generated exclusively by the myeloperoxidase-hydrogen peroxide system (Dahlgren *et al.*, 1985)] was utilised in *ex vivo* studies to assess the neutrophil-specific respiratory burst activity within a mixed population of BALF cells (see Section 2.8.3.4). Thus, the kinetics of PMA- and ZAP-stimulated Lum-DCL in peripheral blood neutrophils were characterized and compared to Luci-DCL responses. This revealed quantitative differences in the CL responses to both ZAP and particularly PMA (Figure 3.4). Analysis of the kinetics of PMA-stimulated Lum-DCL revealed that this was due to a much lower peak CL velocity with a concomitant reduction in total (integral) CL, although nodal times were similar. The kinetics of ZAP-stimulated Luci- and Lum-DCL differed markedly. The luminol signal was bimodal with a very early peak (nodal time 2 min) that waned rapidly (4 min) followed by a gradual increase to a second smaller peak at 60-70 min, indicating two phases of oxidant generation in response to this agonist.

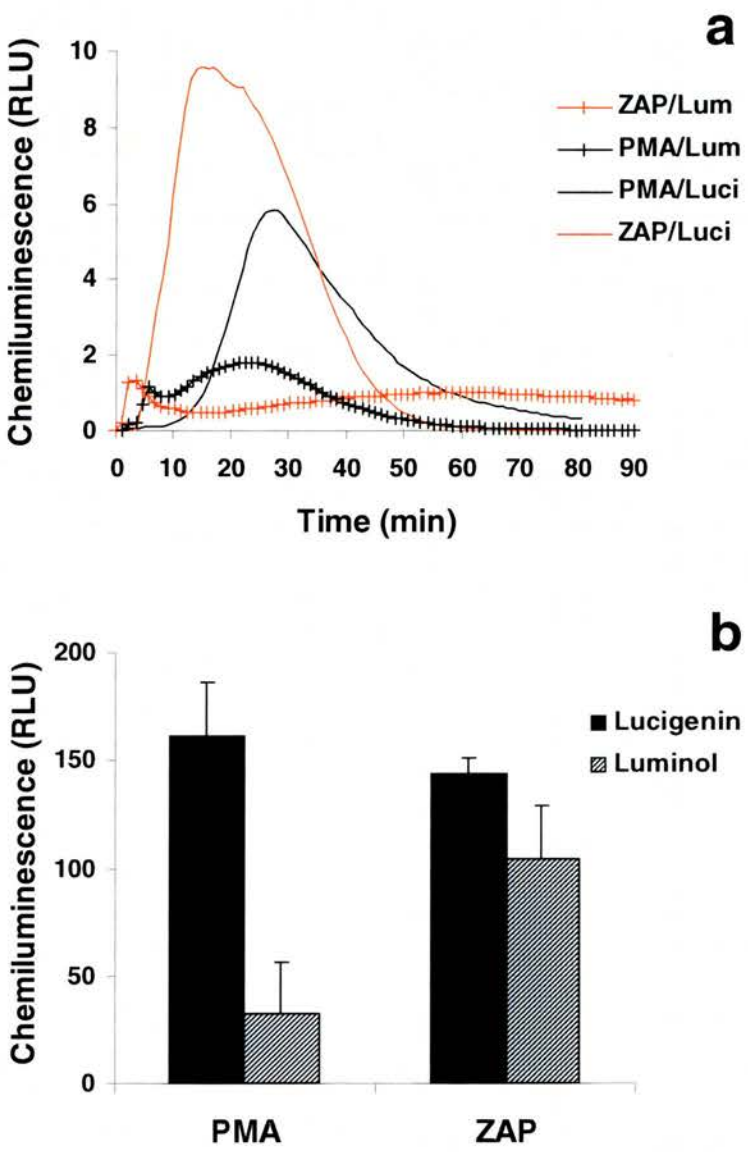


Figure 3.4a,b: Comparison of PMA- and ZAP-stimulated Luci-DCL and Lum-DCL in equine neutrophils.

Cells were stimulated with PMA (100 ng/ml) or ZAP (10%) and CL recorded over 90 min.

a: Time course of PMA (black lines)- and ZAP (red lines)-stimulated Luci (plain lines)- and Lum (hatched lines)-DCL. Data points represent the mean of triplicate determinations from a single experiment representative of 6.

b: Comparison of total (integral) CL in the presence of Lucigenin (closed bars) or Luminol (hatched bars). Values represent mean ± SEM of integral data from 6 separate experiments, each performed in triplicate.

3.2.1.5 Direct effect of LPS, TNF-α, PAF and hrIL-8 on the respiratory burst in equine neutrophils

In these studies, activation of the respiratory burst measured by Luci-DCL was used to define equine neutrophil priming. The ability of LPS, TNF-α, PAF and hrIL-8 to directly stimulate a respiratory burst was determined prior to investigating the priming capacity of these agents (see Section 3.3).

Figure 3.5a shows that LPS was only able to stimulate respiratory burst activity at very high concentrations of (≥10μg/ml). This minor activation may in part reflect the presence of residual plasma factors, which can interact with LPS to trigger neutrophil CL (Benbarek *et al.*, 1998) or may be due to contamination of the smooth LPS preparations used with rough forms of LPS, which are able to directly trigger human neutrophil CL (Kapp *et al.*, 1987).

The ability of human and equine rTNF-α to directly stimulate CL in equine neutrophils was compared. Human rTNF-α stimulated CL at concentrations greater than 10 ng/ml (Figure 3.5a) but this response was highly variable between donors. Equine rTNF-α produced a similarly variable response but displayed activity at substantially lower concentrations (100 pg/ml, Figure 3.5a). The effect of erTNF-α was only tested up to 1 ng/ml because the highest concentration of stock erTNF-α available was 12.5 ng/ml. In spite of regular shaking of the microtitre plate during the luminometer cycle, it is possible that some cell adhesion occurred, which is well recognized to enhance the ability of TNF-α to directly stimulate superoxide anion generation in neutrophils (Schleiffenbaum and Fehr, 1990).

PAF stimulated a very weak but significant CL response only at the highest concentration tested (1 μM), (Figure 3.5b, control 0.1 ± 0.02 RLU; PAF 0.7 ± 0.2 RLU, $n = 3$, $p<0.05$). This compares to data presented by Marr *et al.*, (1997) who observed a small increase in superoxide anion generation in equine neutrophils treated with 10 μM PAF alone. They also reported a significant response to 1 μM PAF following pre-incubation with cytochalasin B, but only in one group of donors tested. PAF (1 μM) alone does not stimulate significant superoxide anion generation in human neutrophils under most assay conditions (Vercellotti *et al.*, 1988; Kitchen *et al.*, 1996b).

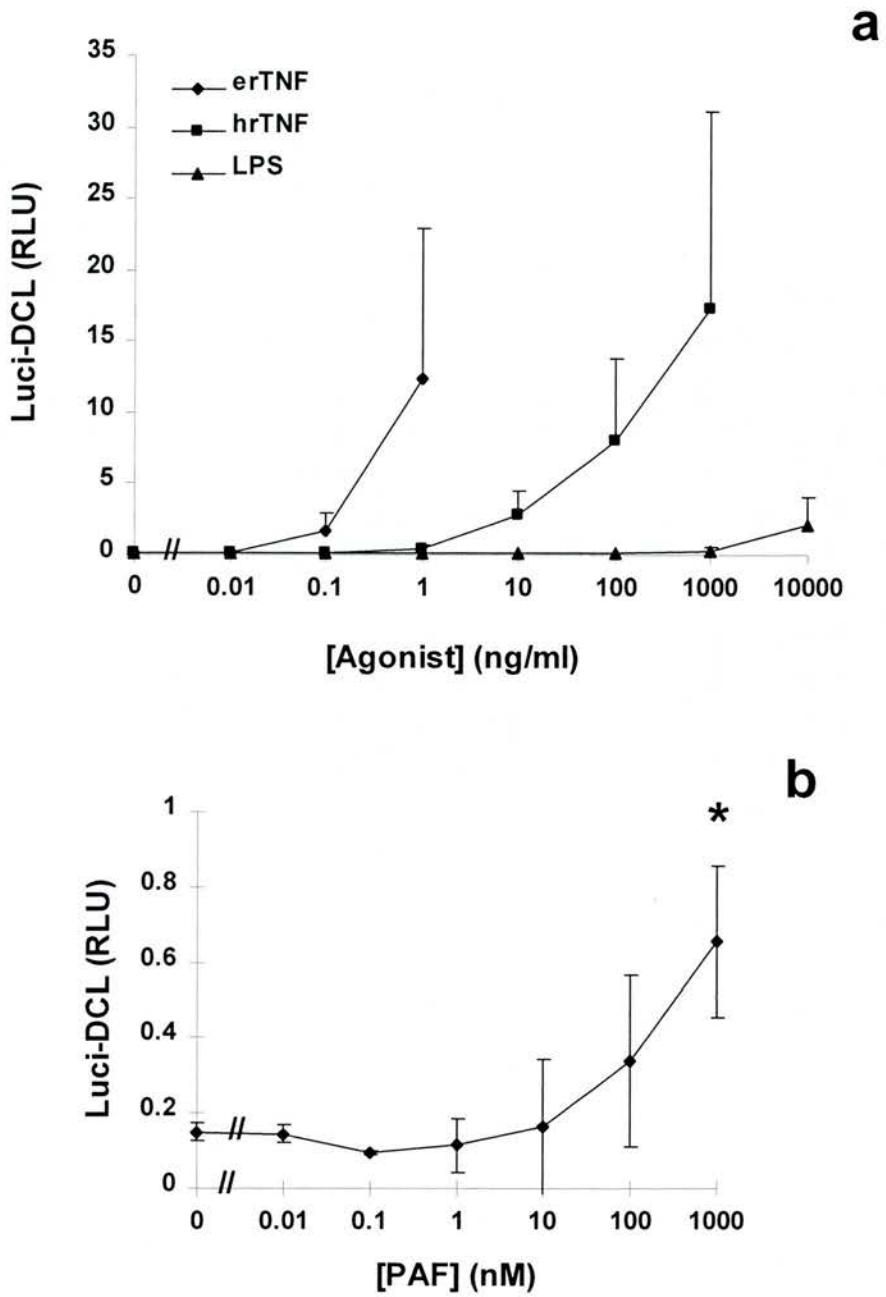


Figure 3.5a,b: Direct effect of LPS, equine and human rTNF-α and PAF on respiratory burst activity in equine neutrophils

Following addition of agonists at the concentrations shown, Luci-DCL was recorded over 60 min. Values represent mean ± SEM of 3 separate experiments each performed in triplicate.

a: Concentration-response curves for equine (diamonds) and hrTNF-α (squares) and LPS-stimulated Luci-DCL (triangles).

b: Concentration-response curve for PAF-stimulated Luci-DCL (* ; $p<0.05$)

Human rIL-8 did not stimulate respiratory burst activity in equine neutrophils at any of the concentrations tested (0.1-1000 ng/ml).

3.2.2 NEUTROPHIL ACTIVATION: CELL POLARIZATION

Neutrophils in the circulation and those isolated under conditions of stable tonicity and free of endotoxin are smooth and spherical (Howard *et al.*, 1990; Fernandez-Segura *et al.*, 1995). Stimulation leads to changes in surface morphology, with the appearance of ridges, ruffles, the extrusion of one or more pseudopodia and ultimately polarization with a front to tail polarity. Changes in cell volume, area and granularity also occur following stimulation (Fernandez-Segura *et al.*, 1995; Keller *et al.*, 1995). These changes result from alterations in the distribution and degree of polymerisation of F-actin (Howard *et al.*, 1990; Bearer, 1993; Fernandez-Segura *et al.*, 1995). The nature of these changes is also governed by the agonist employed, its concentration and the duration of exposure (Keller *et al.*, 1995).
In these studies, freshly isolated equine neutrophils were smooth and spherical when examined under phase contrast microscopy (see Figure 2.4, panel A). Under SEM and TEM they were spherical with short microvilli (SEM and TEM, Figures 3.6a,b) and occasional surface microridges (SEM, Figure 3.6a). As reported previously (Pycock *et al.*, 1988), stimulation with ZAP resulted in dramatic shape change and polarization that was readily apparent under phase contrast microscopy (Figure 2.4, panel B). Also large surface ridges, ruffles, folds and long, granule-free lamellipodia associated with polarization, with an obvious front to tail polarity, were observed under SEM and TEM (Figures 3.6c,d).
Although the flow cytometry assay cannot differentiate the form of shape change induced by an individual agent (Keller *et al.*, 1995), the proportion of cells responding correlates closely with microscopic scoring (see Section 2.3.1.3).

The secretagogue agonists, PMA and ZAP caused a dramatic, concentration-dependent, shape change response in freshly isolated equine neutrophils (% shape change: control, 9.9 ± 2.1%; 10 ng/ml PMA, 83.3 ± 7.1%; 5% ZAP, 91.5 ± 2.6%, 10 min, $p<0.0001$, n=7, Figure 3.7a), with 10 ng/ml PMA and 5% ZAP inducing

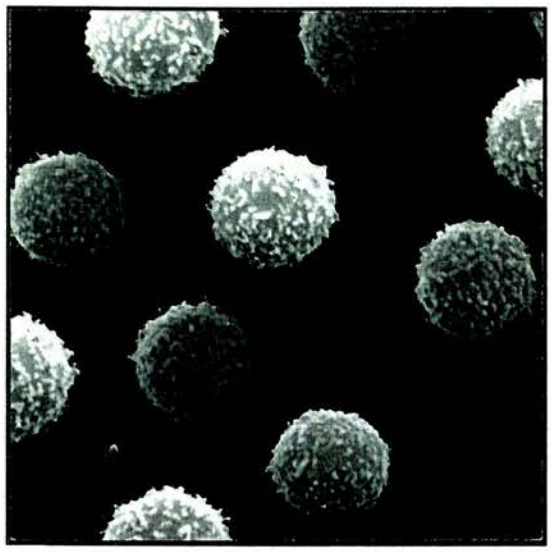


Figure 3.6a: Scanning electron photo-micrograph of freshly isolated neutrophils (Magnification x 11560)

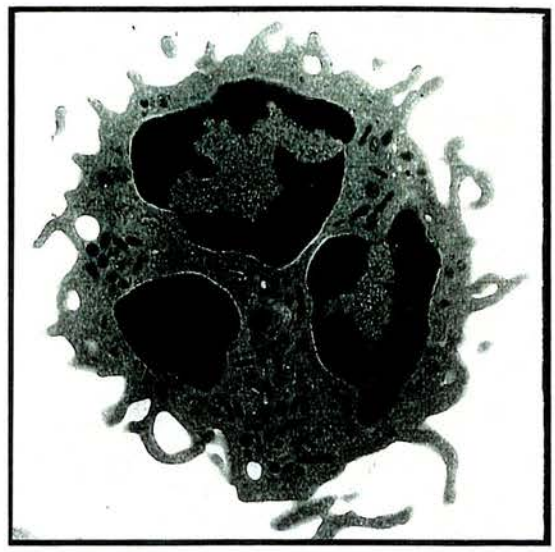


Figure 3.6b:Transmission electron photo-micrograph of freshly isolated neutrophil (Magnification x 13500)

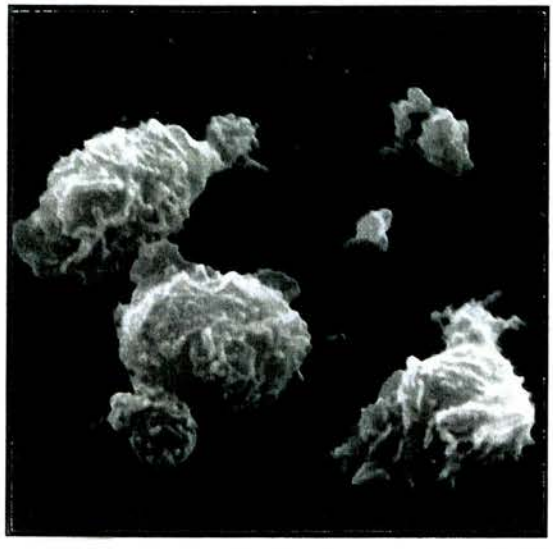


Figure 3.6c: Scanning electron photo-micrograph of ZAP-stimulated (5%,10 min) neutrophils (Magnification x 18500)

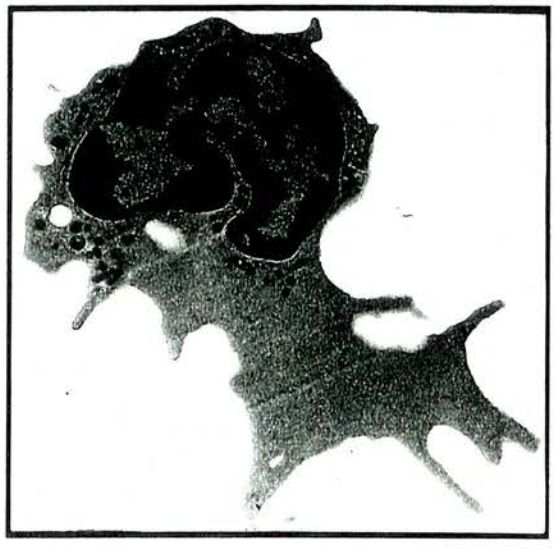


Figure 3.6d:Transmission electron photo-micrograph of ZAP-stimulated (5%, 10min) neutrophil (Magnification x 10270)

Figure 3.6a,b,c,d: Scanning and transmission electron microscopic morphology of quiescent and activated equine neutrophils prepared as described in Section 2.3.2

maximal responses (data not shown). In line with its potent chemotactic effect on equine neutrophils, LTB_4 (10 nM) stimulated significant shape change (% shape change: control, 9.9 ± 2.1%; 10 nM LTB_4, 56.9 ± 8.3%, $p<0.002$, n=7, Figure 3.7a). Human rIL-8 had no effect (% shape change: control, 9.9 ± 2.1%; 1μg/ml hrIL-8, 14.6 ± 4.3%, $p>0.05$, n=7, Figure 3.7a), although the same batch of recombinant cytokine had been shown to be biologically active toward human neutrophils by other workers in the laboratory (Dr L. Bruce, personal communication). This was somewhat disappointing in light of the significant effect of hrIL-8 on equine neutrophil adhesion reported by Marr and colleagues (1999).

Freshly isolated equine neutrophils did not polarize when exposed to 1 μM fMLP (% shape change: control, 9.9 ± 2.1%; fMLP, 11.3 ± 3.0%; $p>0.05$, n = 7, Figure 3.7a). The inability of fMLP to induce cell polarization in equine neutrophils is in marked contrast to its effects in human neutrophils which undergo rapid and concentration-dependent shape change in response to this agent (Kitchen *et al.*, 1996a) (Figure 3.14).

Incubation of cells with either hrTNF-α or PAF induced significant shape change (Figure 3.7b), however, incubation of cells with 1 μg/ml LPS for up to 90 min did not induce a significant response (Figure 3.7b).

Direct stimulation of significant cell polarization by TNF-α and PAF, in the absence of an effect on spontaneous superoxide anion release, is consistent with the priming paradigm recognized in human neutrophils (Haslett *et al.*, 1985; Kitchen *et al.*, 1996b). The failure of 1 μg/ml LPS to stimulate shape change over a 90 min incubation is again in contrast to its effect in human neutrophils, which undergo striking shape change when incubated with as little as 10 ng/ml for 60 min (Haslett *et al.*, 1985).

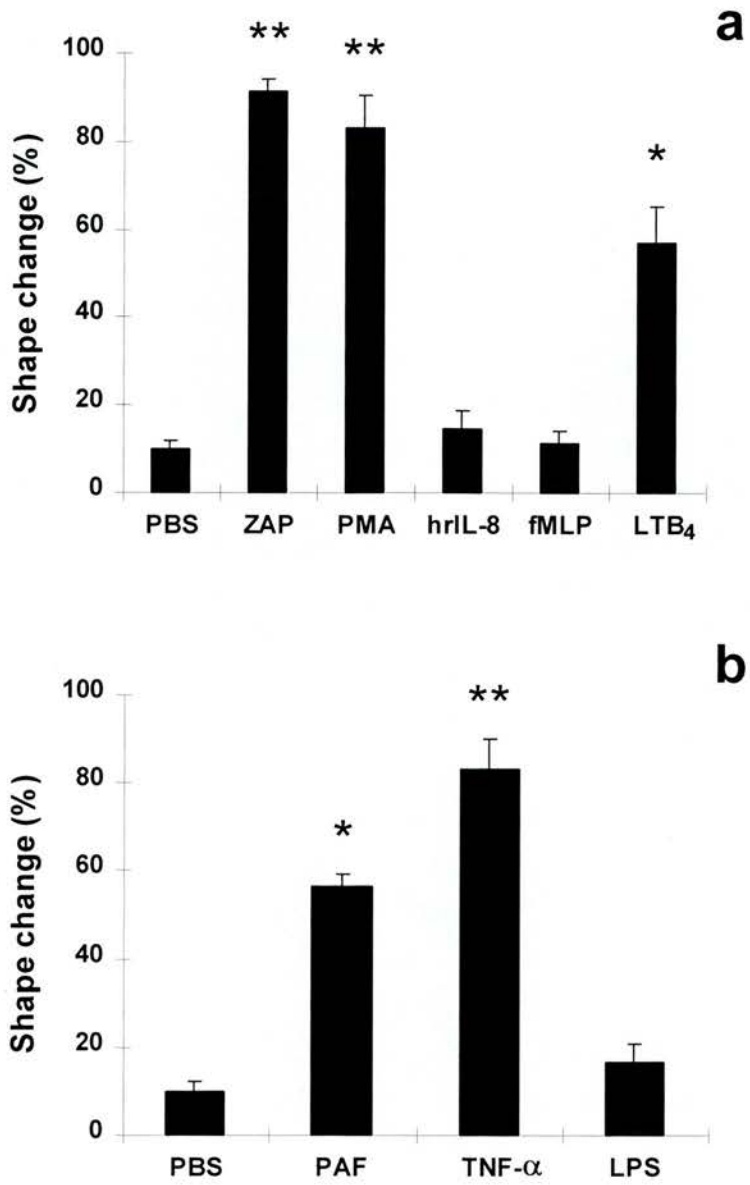


Figure 3.7a,b: Induction of equine neutrophil shape change by secretagogue agonists and priming agents

Neutrophils were incubated for 10 min (LPS, 90 min), fixed with glutaraldehyde and analysed by flow cytometry. Values represent mean ± SEM of 7 separate experiments, each performed in duplicate.

a: Effect of PBS, 5% ZAP, 10 ng/ml PMA, 1 μg/ml hrIL-8, 1 μM fMLP or 10 nM LTB_4.
(* : $p<0.01$; **: $p<0.0001$).

b: Effect of PBS, 1 μM PAF, 200 u/ml hrTNF-α or 1μg/ml LPS.
(* : $p<0.05$; **: $p<0.001$).

3.2.3: PRIMING IN EQUINE NEUTROPHILS

3.2.3.1 Enhancement of ZAP-stimulated respiratory burst by priming agents

Preliminary experiments were designed to characterize ZAP-stimulated Luci-DCL following exposure of equine neutrophils to recognized priming agents to facilitate subsequent assessment of fMLP-stimulated responses. The effect of ZAP was significantly enhanced by pre-treatment with 100 ng/ml LPS + 1% heat-inactivated autologous serum for 90 min (147 ± 10%, n = 8, $p<0.01$, Figure 3.8). Priming increased the initial rate, peak velocity and total (integral) amount of CL generation (see Figure 3.8).

Pre-incubation with both human and erTNF-α (30 min) likewise primed the ZAP-stimulated respiratory burst (hrTNF-α, 200 U/ml: 247 ± 54.5%, n = 4, $p<0.05$, erTNF-α, 1 ng/ml: 227 ± 10%, n = 3, $p<0.05$). However, pre-incubation of equine neutrophils with 1 μM PAF (a concentration shown to induce a significant shape change response, see Section 3.2.2.1) for 10 min had no effect on ZAP-stimulated Luci-DCL. These data confirmed that equine neutrophils were capable of being primed for an enhanced respiratory burst.

3.2.3.2: Induction of fMLP-stimulated respiratory burst by priming agents

Incubation of neutrophils with 1 μM fMLP alone did not stimulate superoxide anion release compared to buffer treated control cells (see Section 3.2.1 and Figures 3.8 & 9). Initially, following LPS + serum priming, a significant and rapid fMLP-induced respiratory burst was apparent (LPS + buffer, 0.4 ± 0.1 peak RLU; LPS + fMLP, 2.4 ± 0.1 RLU, n = 8, $p<0.05$), although this was less marked than that obtained with ZAP (Figure 3.8). This is the first documentation that functional coupling of fMLP receptors in equine neutrophils can stimulate respiratory burst activity. Due to the fast kinetics of this response, a more detailed time-course was examined (Figure 3.9). The kinetics of fMLP-induced Luci-DCL in LPS-primed equine neutrophils demonstrated an extremely brisk superoxide anion response with significant CL detectable as early as 30 sec after fMLP addition, with peak CL occurring between 60-120 sec. Thereafter, respiratory burst activity abated rapidly with virtually complete loss of the response by 4 min. This pattern of superoxide anion generation

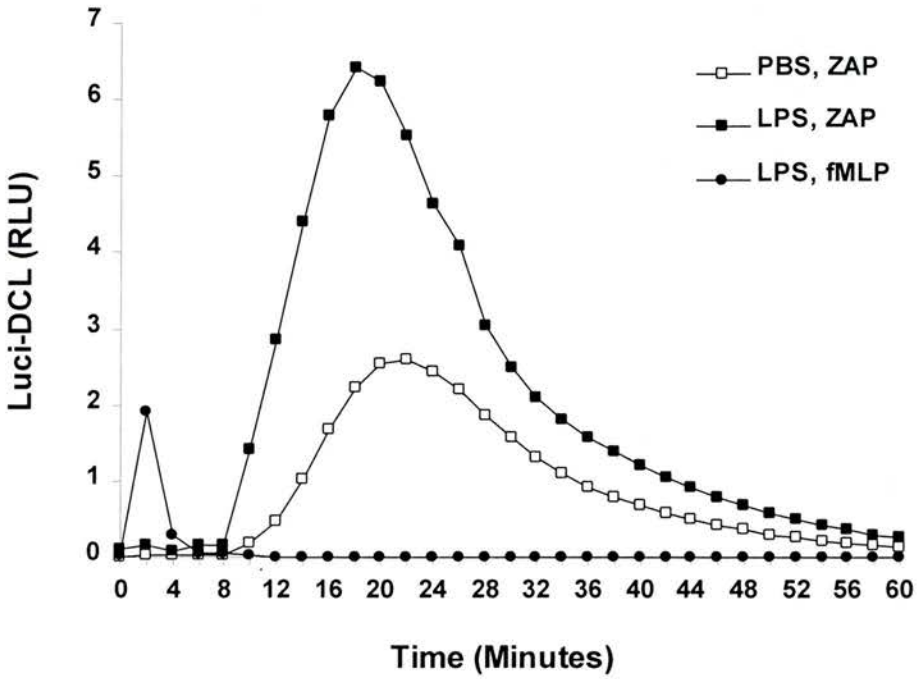


Figure 3.8: Effect of LPS priming on ZAP- and fMLP-stimulated respiratory burst in equine neutrophils

Neutrophils were incubated with PBS or 100 ng/ml LPS + 1% heat-inactivated autologous serum for 90 min prior to stimulation with PBS, 10% v/v ZAP or 100 nM fMLP. Luci-DCL was monitored over 60 min. The Luci-DCL signal generated by PBS or LPS pre-treated cells stimulated with PBS, and PBS pre-treated cells stimulated with fMLP was not significantly different to baseline; these values have been omitted for clarity. Values represent mean of triplicate measurements from a single experiment, representative of eight.

is very similar to that reported for human neutrophils (Dahlgren *et al.*, 1985). The optimum conditions for LPS priming were then investigated. The effect of LPS on fMLP-stimulated Luci-DCL was observed to be both time- and concentration-dependent (Figures 3.10a-c). Addition of 1% heat-inactivated autologous serum significantly potentiated both the temporal initiation of the response and the sensitivity of the cells to LPS (Figures 3.10a-c). Cells co-incubated for 30 min with heat-inactivated autologous serum and LPS at concentrations ≥ 100 ng/ml became responsive to fMLP (Figure 3.10a). In the presence of serum, the maximal response was observed by 60 min with a mean EC_{50} of 19.1 ± 4.7 ng/ml for LPS (Figure 3.10b). In the absence of serum, a 90 min LPS-incubation was required to achieve an equivalent maximal response, albeit with the cells remaining less sensitive to LPS (+ serum; EC_{50} of 11.7 ± 2.6 ng/ml, - serum; EC_{50} of 71.5 ± 2.7 ng/ml, Figure 3.10c). Of note, the true serum dependence of the LPS priming effect in equine neutrophils shown above, was only revealed when an extensive initial wash protocol was employed (see Section 2.1.2), suggesting that a significant amount of LPS-binding protein (LBP) or its equine equivalent remains tightly associated with neutrophils, when they are inadequately washed.

In human neutrophils, LPS priming for enhanced superoxide anion generation is well recognized to be largely serum-dependent, due to a requirement for LBP in the cellular recognition of LPS (Aida and Pabst, 1990; Shapira *et al.*, 1995); these data would suggest the involvement of a similar LBP/CD14 mechanism for LPS priming in equine neutrophils. Indeed, we demonstrated that human serum could substitute for equine serum (species' autologous sera prepared freshly) in augmenting the LPS priming of equine neutrophils and *vice versa* (Figure 3.11a,b). In both cross over experiments, using a 30 min pre-incubation period, human serum was significantly more effective in its enhancement of LPS priming of both human ($p<0.05$, repeated measures ANOVA) and equine cells ($p<0.01$) than equine serum. This effect was also apparent for human cells after 60 min ($p<0.05$). This could indicate that there are qualitative and/or quantitative differences in the lipopolysaccharide binding proteins present in normal human serum compared to equine serum and may, in part, explain the lower sensitivity of equine cells to LPS under these assay conditions. This warrants further detailed investigation. The presence of LBP has not previously

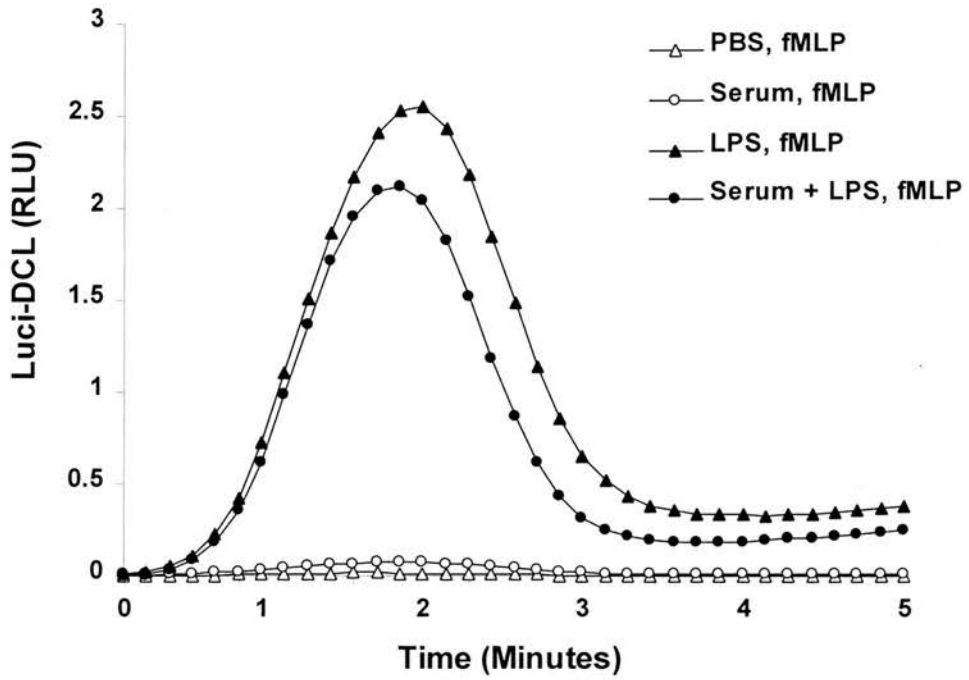


Figure 3.9: Time course of fMLP-stimulated Luci-DCL

Time-course of fMLP-stimulated Luci-DCL following a 90 min pre-treatment with PBS or 1 µg/ml LPS both performed in the presence and absence of 1% heat-inactivated autologous serum. Values represent mean ± SEM of 3 separate experiments each performed in triplicate. SEM values were less than 10% for all data points.

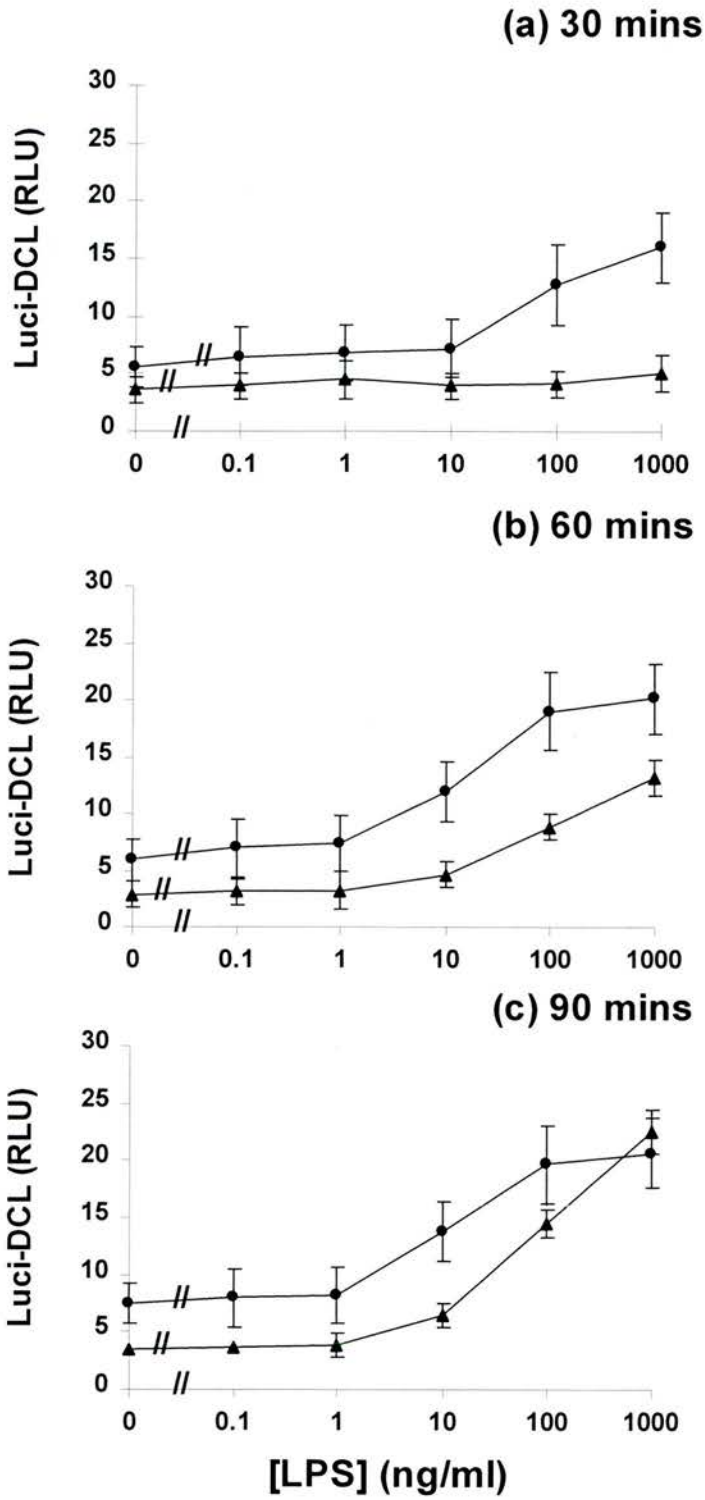


Figure 3.10a,b,c: LPS priming in equine neutrophils: Concentration-response curves
Cells were stimulated with fMLP (1 μM) after incubation with buffer or LPS (0.1 –1000 ng/ml) in the presence (circles) or absence (triangles) of 1% serum for (a) 30, (b) 60 or (c) 90 min. Values represent mean ± SEM of five separate experiments, each performed in duplicate.

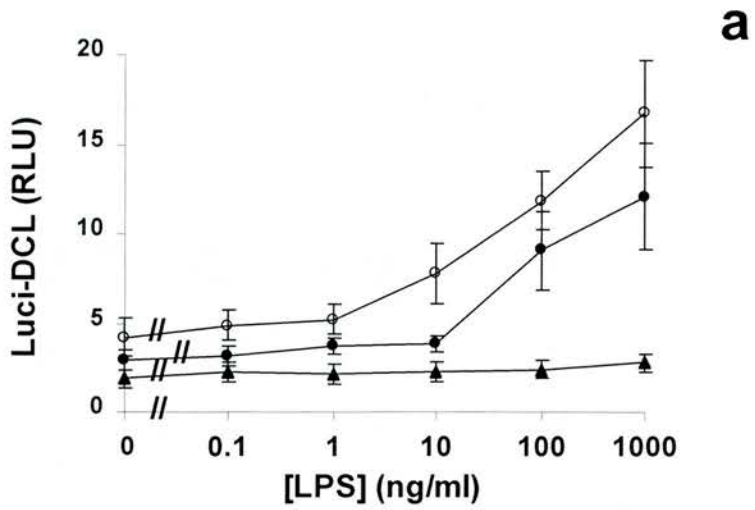


Figure 3.11a: Human serum enhances LPS priming of equine neutrophils
Equine neutrophils were stimulated with fMLP (1μM) after incubation with buffer or LPS (0.1 –1000 ng/ml) in the absence (triangles) or presence of 1% equine (closed circles) or human (open circles) serum for 30 min. Values represent mean ± SEM of three separate experiments, each performed in duplicate.

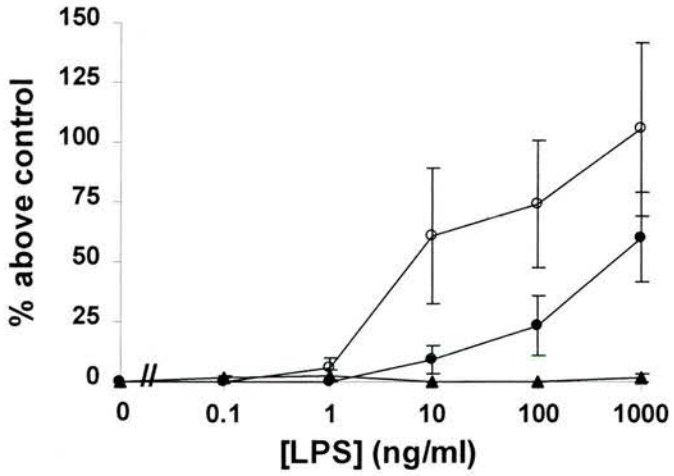


Figure 3.11a,b: Equine serum enhances LPS priming of human neutrophils
Human neutrophils were stimulated with fMLP (1μM) after incubation with buffer or LPS (0.1–1000 ng/ml) in the absence (triangles) or presence of 1% equine (closed circles) or human serum (open circles) for 30 min. Values represent mean ± SEM of % of response in the absence of LPS in three separate experiments, each performed in duplicate.

been studied in the horse and consequently, appropriate reagents for its quantitative analysis are, as yet, not available. In other species the complex of LPS and lipopolysaccharide binding protein is recognized by the glycosyl-phosphatidylinositol-anchored membrane protein CD14 (Wright *et al.*, 1990). Human neutrophils in whole blood express 1,900 – 4,400 CD14 receptors, with no change in expression following neutrophil isolation (Antal-Szalmos *et al.*, 1997). To investigate this mechanism further, equine neutrophils were screened for their ability to bind a panel of currently available anti-human CD14 monoclonal antibodies. Neutrophils and mixed mononuclear cells (1×10^6 cells/ml) harvested from Percoll gradients were washed then resuspended in 50 µl of one of a panel of anti-human CD14 or isotype control antibodies (dilutions in brackets) and incubated (30 min) on ice in a 96-well plate. Three fluorescent conjugates were screened, TUK 4 (1/10, DAKO, High Wycombe, UK), MY 4 (1/20, Coulter Electronics Ltd., Luton, UK) and a control goat anti-mouse polyclonal $F(Ab')^2$ (1/20, DAKO). The unconjugated antibodies, MR3 (1/50), NC 2 (1/20), UCHM1 (1/10, SAPU, Carluke, UK), SAPU CD14 (1/20, SAPU), 61D3 (1/20) and a control MOPC IgG1 (1/20, EACC) were, further incubated on ice (30 min) with a secondary fluorescent antibody conjugate. MR3, NC2 and 61D3 were gifts from Dr Ian Dransfield, Rayne Laboratory, University of Edinburgh. Human monocytes were incubated with TUK 4, MY 4, UCHM1 and SAPU CD14 to generate positive control data. Antibody binding was analysed by flow cytometry (EPICS Profile II). No CD14 cross-reactivity was observed with equine neutrophils, although positive staining of human monocytes by TUK 4, MY 4, UCHM 1 and SAPU CD14 was detected (data not shown). Hence the presence or absence of CD14 on these cells and a more detailed dissection of this mechanism, remains to be explored in the horse.

The fMLP effect on Luci-DCL in LPS primed neutrophils was also shown to be concentration-dependent with an EC_{50} of 10.2 ± 3.9 nM (Figure 3.12). This appears to be very similar to that demonstrated graphically by Snyderman and Pike (1981) for fMLP-stimulated lysozyme release from cytochalasin B-treated equine neutrophils. This value is less than that reported for human blood neutrophils (91 ± 14 nM) and substantially lower than for guinea pig blood neutrophils (32 µM)

(Zimmerli *et al.*, 1986). Interestingly, however, this EC_{50} for equine neutrophils is of the same order of magnitude as for human (14 ± 19 nM) and guinea pig (5.8 nM) neutrophils functionally primed *in vivo*, by exudation (Zimmerli *et al.*, 1986).

To examine whether LPS was unique in its ability to induce functional coupling of the fMLP receptor in equine cells, the effects of PAF and hrTNF-α were also investigated (Figure 3.13). Subsequently, the concentration dependence of the priming effects of PAF and human and erTNF-α were studied. In contrast to its effect in human cells (Kitchen *et al.*, 1996a; Kitchen *et al.*, 1996b) and, despite demonstrating a significant concentration-dependent shape change effect, PAF appeared to have a very weak effect in augmenting fMLP-stimulated superoxide anion generation in equine neutrophils (Figure 3.13, Table 3.1).

Human rTNF-α induced a major up-regulation of fMLP-induced Luci-DCL (Figure 3.13, Table 3.1). However, at the concentration used in the initial experiments shown in Figure 3.13, hrTNF-α (200 U/ml) treatment alone stimulated a significant Luci-DCL response, indicating that this cytokine was also acting as a modest direct secretagogue. Further experiments, including comparison with erTNF-α, demonstrated concentration-dependent and comparable priming of the fMLP response with both recombinant cytokines (Table 3.1).

The potential for hrIL-8 to prime equine neutrophils was also examined by incubating cells with 1 μg/ml hrIL-8 (30 min, 37°C) prior to stimulation with fMLP (1μM). In agreement with previous data showing no shape change or secretagogue effects of hrIL-8 in equine neutrophils, no significant CL signal was observed (data not shown).

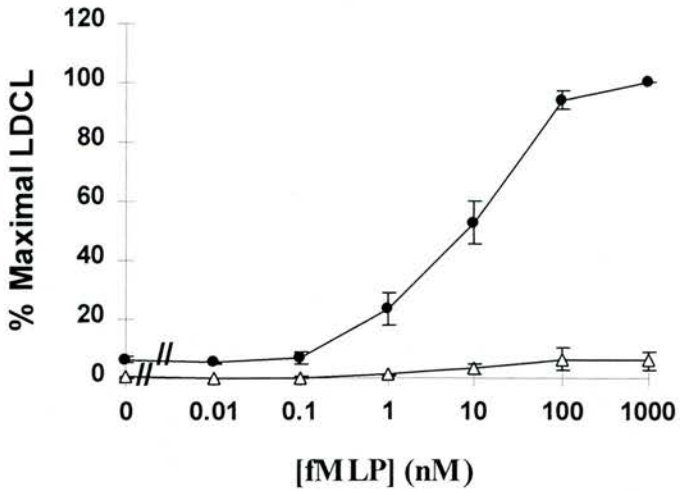


Figure 3.12: Concentration-response curve for fMLP-stimulated Luci-DCL in LPS primed equine neutrophils

Equine neutrophils were pre-incubated with PBS (open triangles) or 1 µg/ml LPS + 1% serum (closed circles) for 90 min prior to addition of lucigenin and stimulation with 0.01 - 1000 nM fMLP. Luci-DCL was recorded at 8 s intervals for 5 min. Values are expressed as % of maximum response to 1000 nM fMLP and represent mean ± SEM of triplicate determinations from a single experiment representative of three (mean maximal Luci-DCL 9.9 ± 0.6 RLU).

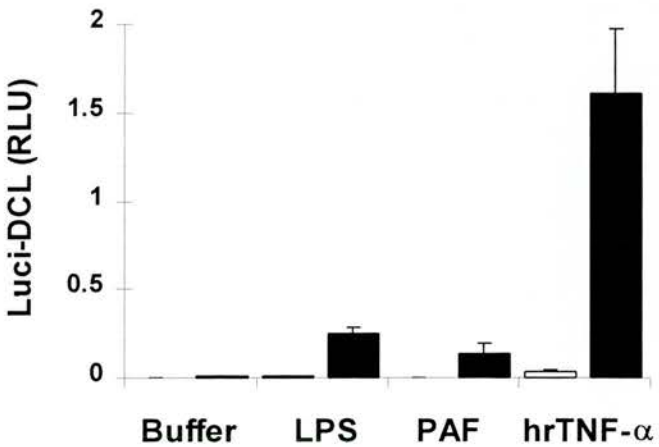


Figure 3.13: Comparative priming effect of LPS, PAF and hrTNF-α on fMLP-stimulated Luci-DCL in equine neutrophils

Neutrophils were pre-incubated with LPS (1 µg/ml, 90 min) + 1% heat-inactivated autologous serum, PAF (100 nM, 10 min), hrTNF-α (200 U/ml, 30 min) or PBS (90 min) prior to stimulation with PBS (open bars, left of pair) or 100 nM fMLP (closed bars, right of pair). CL was recorded at 8s intervals for 5 min. Peak values presented as mean ± SEM of three separate experiments, each performed in triplicate.

Priming agent (Concentration and incubation period)	**Maximal CL (RLU)** (mean ± SEM)	**EC_{50}** (mean ± SEM)	**Experiments** (n)
LPS + 1% serum (1 μg/ml, 60 min)	19.1 ± 3.5	19.1 ± 4.7 ng/ml	5
PAF (1 μM, 10 min)	3.1 ± 1.1	10.4 ± 2.6 nM	4
hrTNF-α (10 ng/ml, 30 min)	64.4 ± 7.9	1.2 ± 0.5 ng/ml	3
hrTNF-α (1 ng/ml, 30 min)	40.5 ± 12.0	1.2 ± 0.5 ng/ml	3
erTNF-α (1 ng/ml, 30 min)	29.9 ± 8.7	0.21 ± 0.03 ng/ml	3

Table 3.1: Capacity of different priming agents to induce functional coupling of fMLP receptors in equine neutrophils

Cells were incubated with priming agents (concentrations and incubation periods as indicated in column 1) prior to stimulation with fMLP (1 μM). Luci-DCL was recorded over 5 min. The incubation conditions in column 1 resulted in the maximal Luci-DCL responses in column 2 with an EC_{50} as quoted in column 3. Pre-incubation with PBS only prior to stimulation with fMLP (1 μM) gave a mean (range) Luci-DCL response of 1.37 (0.2 – 2.9) RLU. Values represent mean ± SEM of n separate experiments each performed in duplicate.

3.2.3.3 Induction of fMLP-stimulated shape change by LPS

In contrast to the effects of ZAP (5% v/v, 10 min), which caused a dramatic shape change response, freshly isolated equine neutrophils did not polarize when exposed to 1 μM fMLP for the same period of time (see Section 3.2.2.1 and Figures 3.7a & 3.14). Likewise, incubation of cells with buffer alone for 90 min did not influence this lack of response to fMLP (% shape change: control, 12.9 ± 1.5%; fMLP, 16.8 ± 2.1; n = 4, p >0.05, Figure 3.14). However, following treatment of cells with 1 μg/ml LPS for 90 min, fMLP induced a modest but significant increase in equine neutrophil shape change (% shape change: LPS 14.5 ± 3.8%; LPS + 1 μM fMLP, 29.4 ± 5.0%; n = 4, p <0.05, Figure 3.14) with an EC_{50} for fMLP of 1.9 ± 0.9 nM (data not shown). In marked contrast, human neutrophils undergo a rapid and concentration-dependent shape change response to fMLP alone (Kitchen *et al.*, 1996a) (Figure 3.14).

The effect of LPS priming on fMLP-stimulated equine neutrophil shape change was somewhat variable between donors; a small population of shape-changed cells being observed following incubation with LPS and serum in two donors, consistent with the human neutrophil/LPS priming paradigm. Moreover, in these two experiments the cells subsequently showed a much more dramatic, concentration-dependent polarization response to fMLP (% shape change: LPS/serum, 24.2 and 29.1%; LPS/serum + 1 μM fMLP, 46.0 and 86.8%, respectively). However, the variance between donors precluded a significant statistical analysis. Ideally further experiments should be performed to elucidate this effect in detail. However, such variation may be associated with individual donor phenotype in terms of LPS sensitivity, as has been reproducibly demonstrated in normal human subjects following LPS inhalation (Kline *et al.*, 1999). Furthermore, the reproducible variation in clinical response was mirrored in the cytokine secretory response of isolated alveolar macrophages and blood monocytes. Further studies will be required to establish similar phenotypic variation in neutrophil responses and whether this has an underlying genetic basis.

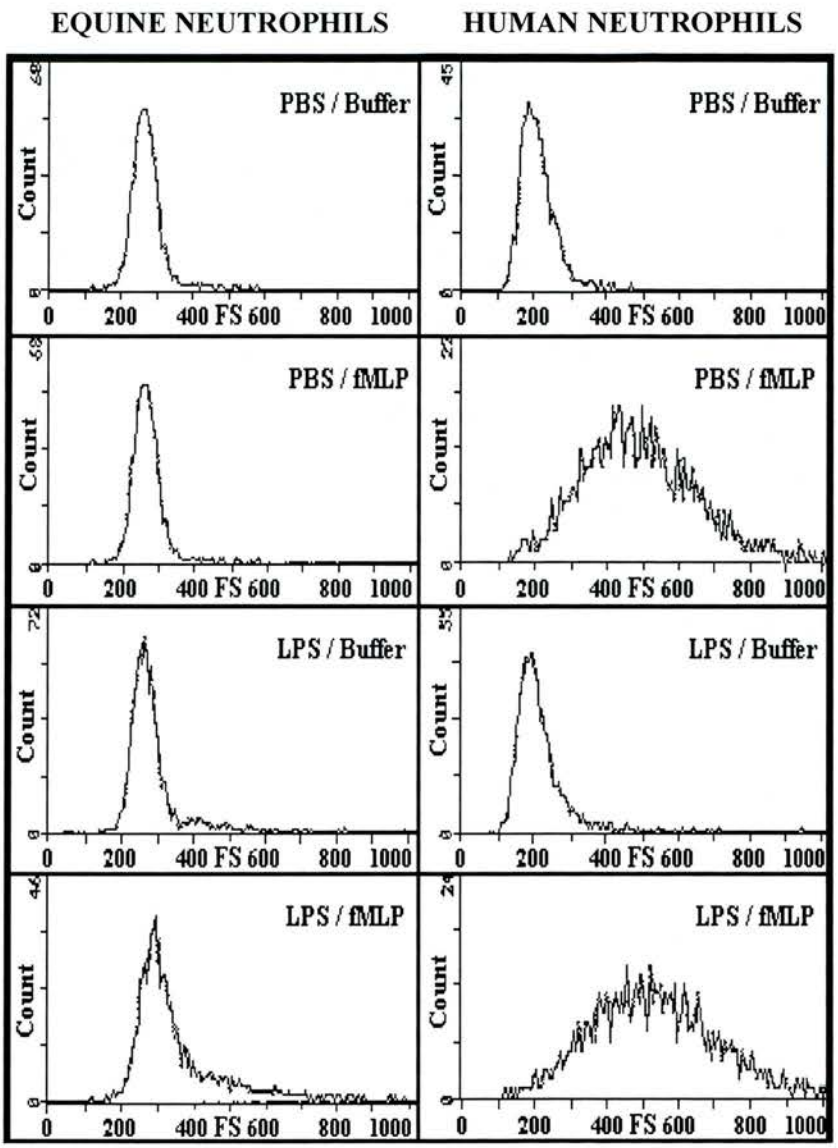


Figure 3.14: Effect of LPS priming on fMLP-induced shape change in equine and human neutrophils

Equine or human neutrophils were pre-incubated for 90 min with PBS or 1 µg/ml LPS prior to stimulation with buffer or 100 nM fMLP for 10 min. Representative flow cytometry histograms of glutaraldehyde-fixed equine (left panels) and human (right panels) neutrophils are shown (pre-incubation / stimulation conditions are indicated within the panels). Data represent mean forward light scatter (x axis) plotted against cell number (y axis). Similar data were obtained from 3 additional experiments, each performed in duplicate.

3.2.3.4 LPS fails to induce fMLP-mediated chemotaxis in equine neutrophils

Analysis of chemotaxis data failed to demonstrate any chemotactic response of unprimed equine neutrophils to 0.01-1000 nM fMLP (Figure 3.15). In contrast to the above superoxide anion generation and cell polarization data, pre-treatment of equine neutrophils with 1 μg/ml LPS (+ serum) for 90 min did not facilitate fMLP-directed chemotaxis (Figure 3.15). Positive control chemotaxis data were obtained both in equine cells using ZAP (Figure 3.14) and in human cells using fMLP (data not shown).

3.2.3.5 [^{3}H]fMLP radioligand binding studies

To determine that the effect of fMLP was likely to be receptor-mediated, radioligand binding studies were undertaken to quantify fMLP receptor number and affinity in equine neutrophils and to examine the effects of LPS priming on receptor expression. Scatchard analysis of [^{3}H]fMLP binding to equine neutrophils demonstrated a single set of high affinity fMLP receptors (K_d 9.3 x 10^{-11}M), with a mean of 660 ± 159 (n = 3) receptors per cell (Figure 3.16a). This compares to data obtained in parallel experiments in human neutrophils, where Scatchard analysis demonstrated a two receptor site model with a mean of 36,000 low affinity receptors (K_d 3.2 x 10^{-8}M) and 2,100 high affinity receptors (K_d 9.2 x 10^{-10}M) per cell (Figure 3.16b), consistent with previous reports (O'Flaherty *et al.*, 1991). Following LPS (+ serum) priming of equine cells (1 μg/ml, 90 min), a similar single receptor site model fit was again obtained (K_d 1.4 x 10^{-10}M), but with a significant 2.5 fold increase ($p<0.05$, n = 3) in total receptor numbers to 1400 ± 398 per cell (Figure 3.16a).

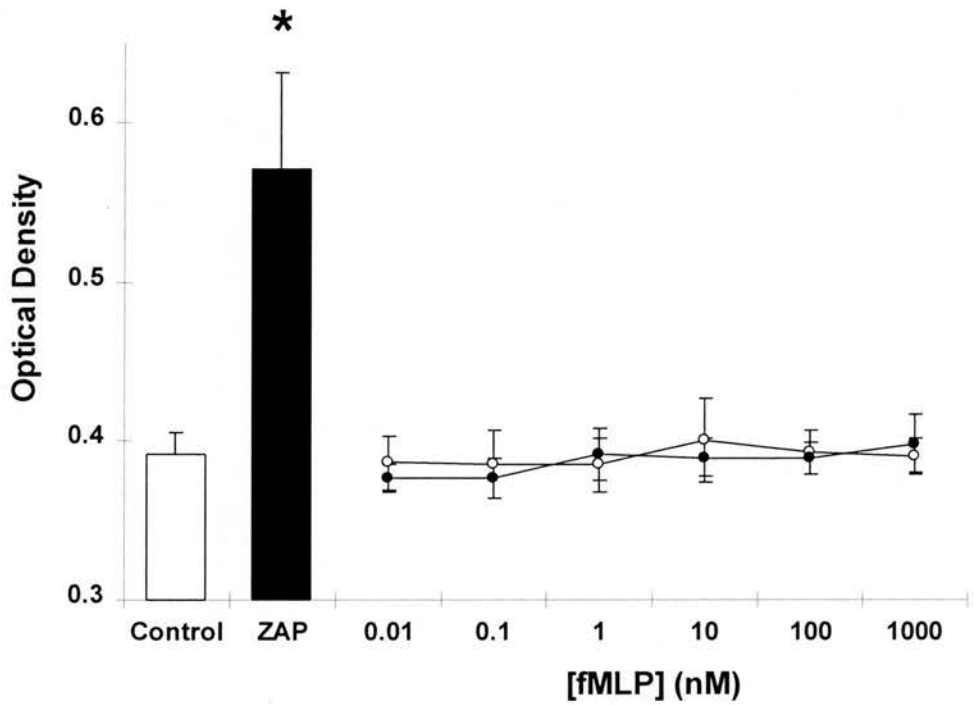


Figure 3.15: Failure of LPS to induce fMLP-stimulated chemotaxis

Equine neutrophils were incubated for 90 min with PBS + 1% serum in the presence (closed circles) or absence (open circles) of 1 µg/ml LPS. Chemotaxis towards fMLP (0.01 - 1000 nM) or ZAP (10% v/v, no LPS pre-incubation) was assayed using a Neuroprobe 96-well chemotaxis chamber incorporating a 5 µm pore filter which was stained with Diff-Quick prior to measurement of optical density. Values represent mean ± SEM of 5 separate experiments, each performed in triplicate. (* : $p < 0.01$).

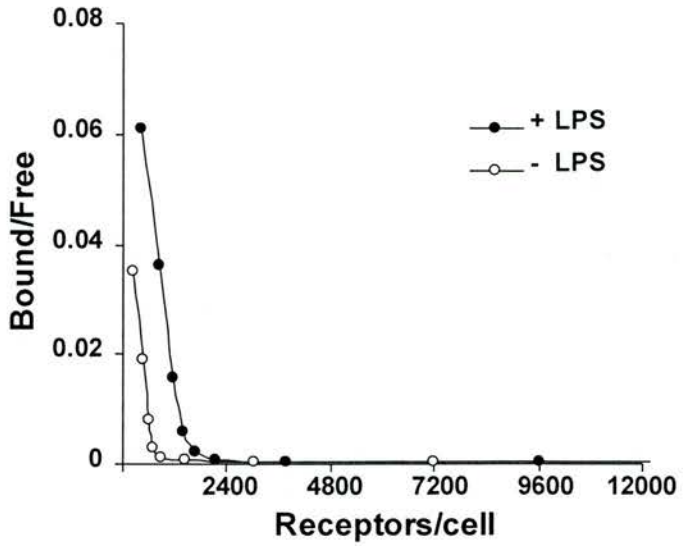


Figure 3.16a: Scatchard analysis of [^{3}H]fMLP binding to equine neutrophils
Equine neutrophils were pre-incubated for 90 min in PBS and 1% heat-inactivated autologous serum in the presence (closed circles) or absence (open circles) of 1 µg/ml LPS prior to assessment of [^{3}H]fMLP binding as detailed in Section 2.5. Scatchard transformed data are shown where each point represents the mean of three experiments each performed in triplicate.

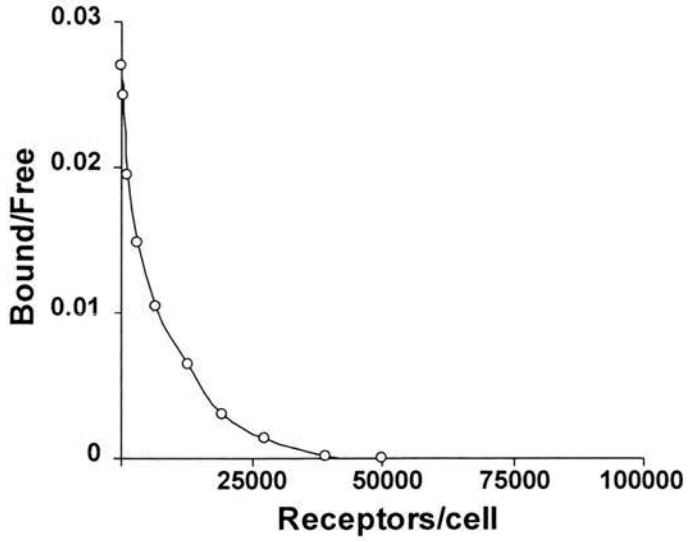


Figure 3.16b: Scatchard analysis of [^{3}H]fMLP binding to human neutrophils
Human neutrophils were pre-incubated for 90 min in PBS and 1% heat-inactivated autologous serum prior to assessment of [^{3}H]fMLP binding as detailed in the Section 2.5. Scatchard transformed data are shown where each point represents the mean of three experiments each performed in triplicate.

3.2.3.6 Failure of priming to enhance PMA-stimulated chemiluminescence

PMA is a potent stimulus of the respiratory burst in equine neutrophils (Section 3.2.1.1), and although it is not a physiological secretagogue, this response has been reported to be enhanced by certain priming agents and has been has been widely used in the investigation of human neutrophil priming. Enhancement of PMA-stimulated superoxide anion generation has been recognized following priming with LPS in both human (Bochsler *et al.*, 1990) and equine cells (Bochsler *et al.*, 1992) and with PAF (Gay *et al.*, 1986; Vercellotti *et al.*, 1988) and TNF-α (Berkow *et al.*, 1987) in human cells. However, more recent studies found no difference in the response of human neutrophils to PMA when primed with hrTNF-α (Bajaj *et al.*, 1992). When equine neutrophils were primed with these agents using the conditions described in Table 3.1, no enhancement of PMA-stimulated Luci-DCL was observed in comparison to cells incubated with buffer alone (n = 3). In fact, after pre-incubation with LPS, PAF and hrTNF-α, there was a trend toward an inhibition of Luci-DCL in comparison to the PMA-induced response observed in PBS-incubated cells. However, this did not reach statistical significance with any of the priming agents (data not shown). Although none of the studies referred to above used CL to quantify the respiratory burst, enhancement of PMA-stimulated Lum-DCL has been reported after pre-incubation of human neutrophils with sub-activating concentrations of fMLP and C5a (Bender *et al.*, 1983). Moreover, lucigenin should be more sensitive than luminol to an enhanced release of superoxide anions following priming. This possible biochemical anomaly in the PMA-stimulated response in equine neutrophils primed *in vitro* warrants further study, particularly in light of the enhanced CL response to PMA in blood neutrophils harvested from COPD-susceptible horses 24 h after hay/straw challenge (see Section 5.2.8.3).

3.3 DISCUSSION

Prediction of the functional consequences of neutrophil behaviour *in vivo* by extrapolation from models of priming and activation in isolated populations of cells, pre-supposes that their function equates with that of cells within the circulation. The

importance of employing a neutrophil isolation technique that produces a highly purified population of minimally primed cells has been established as a crucial factor in studying the true responsiveness of human neutrophils (Haslett *et al.*, 1985; Watson *et al.*, 1992; Pabst, 1994), but this factor has not been previously addressed in equine leukocytes.

In view of the absent, extremely weak or inconsistent *in vitro* (Camp and Leid, 1982; Zinkl and Brown, 1982; Sedgwick *et al.*, 1987; Benbarek *et al.*, 1996) and *in vivo* (McEwen and Lumsden, 1991) responses elicited by fMLP in the horse, the lack of effect of fMLP on superoxide anion release in this species was attributed to a lack of any functional fMLP receptor coupling. Indeed, in agreement with this previously held view, we were unable to detect any chemotactic or polarization response to fMLP in unprimed equine neutrophils despite the very clear mobility effects of this agent in human neutrophils, which are observed even in "quiescent", unprimed cells (Qu *et al.*, 1995; Condliffe *et al.*, 1996; Kitchen *et al.*, 1996a).

The data presented here regarding the ability of priming agents to induce functional coupling of fMLP receptors in equine neutrophils, the minimal basal shape change and fMLP-stimulated Luci-DCL that we observed in freshly isolated cells supports the choice of the two-step plasma-Percoll isolation method employed. Indeed, previous reports of fMLP-stimulated secretion in freshly isolated equine neutrophils (Snyderman and Pike, 1980; Snyderman and Goetzl, 1981; Bertram, 1985) raises the possibility that the neutrophil isolation techniques used in these studies caused some degree of priming. Moreover, the fMLP-stimulated lysozyme release demonstrated by Snyderman and Pike (1980) followed treatment with cytochalasin B, a non-physiological priming agent. Recently, we have shown that equine neutrophils do not release elastase in response to fMLP alone but show a major degranulation response following LPS priming (Dr. M.P. Dagleish and T.J. Brazil, unpublished observations); these data again support the notion that priming is a prerequisite for functional coupling of fMLP receptors in equine neutrophils. Furthermore, the non-reproducible CL response of equine neutrophils to fMLP observed by Benbarek and co-workers (1996) may have been related to a variable state of priming in different batches of isolated cells.

However, even the most physiologically stringent isolation technique is unlikely to leave cells entirely unperturbed, with adhesion molecule expression, in particular known to be especially sensitive to the rigours of neutrophil isolation (Kuijpers *et al.*, 1991; Watson *et al.*, 1992). Until specific reagents for the recognition of equine neutrophil surface markers are developed, progress toward an optimal isolation method for these cells will be hampered. Perhaps the most robust and physiological benchmark for assessment of an isolation technique is the behaviour of isolated cells following re-infusion into the donor animal. Indeed, the modification of this plasma-Percoll method for rabbit neutrophils produces cells that, following ^{111}In-tropolonate labelling and re-infusion, circulate and respond to pharmacological induction of neutrophilia and neutropaenia in a manner identical to that of unmanipulated cells (Haslett *et al.*, 1987). Assessment of unstimulated CL generation following cell isolation and discarding cells where there was evidence of basal activation was a further important quality control measure employed in the current characterization of equine neutrophil priming.

It is important to recognize that Luci- and Lum-DCL responses reflect different aspects of respiratory burst activity in neutrophils and hence are not directly comparable. Lucigenin, a relatively large molecule (510 Daltons) remains extracellular and hence detects only externally secreted reactive oxygen species (predominantly superoxide anions) whereas luminol (177 Daltons) is also able to penetrate cells and interacts almost exclusively with hydrogen peroxide and its breakdown products catalysed by myeloperoxidase (Dahlgren *et al.*, 1985). However, comparisons between data from the different time points in *ex vivo* experiments remain valid, providing that assay conditions with each luminigenic probe are constant.

The CL response we observed following PMA stimulation is consistent with data reported previously in both equine and human neutrophils (Aniansson *et al.*, 1984; Allen, 1986; Benbarek *et al.*, 1996). Burgener and colleagues (1998) studied homologous ZAS-stimulated Lum-DCL in canine neutrophils and observed a small peak of activity at 30 s, but they only recorded CL for 2 min, precluding comparison of kinetics to those observed here. The bimodal ZAP-stimulated Lum-DCL seen in equine cells may have similar origins to that observed in human neutrophils in

response to fMLP; the initial peak being associated with extracellular release of MPO and H_2O_2 followed by a delayed secondary peak of CL generated intracellularly (Briheim *et al.*, 1984). However, measurement of fMLP-stimulated Lum-DCL in equine neutrophils during the *ex vivo* experiments did not reveal a bimodal response. The biochemical and physico-chemical mechanisms governing these differences between species and agonists are complex (Benbarek *et al.*,1996; Harbecke *et al.*, 1996) and warrant further investigation.

The highly sensitive CL assay employed in this study demonstrated that fMLP-stimulated Luci-DCL could be observed but only following LPS pre-treatment i.e. consistent with this being a primed response (Guthrie *et al.*, 1984; Haslett *et al.*, 1985). fMLP-stimulated Luci-DCL was also observed following pre-incubation with PAF and TNF-α, both well recognized neutrophil priming agents in man (Kitchen *et al.*, 1996a; Kitchen *et al.*, 1996b), however, PAF primed this response only very weakly in equine neutrophils. Furthermore, this priming effect was not restricted to fMLP, since LPS and TNF-α also enhanced ZAP-stimulated Luci-DCL.

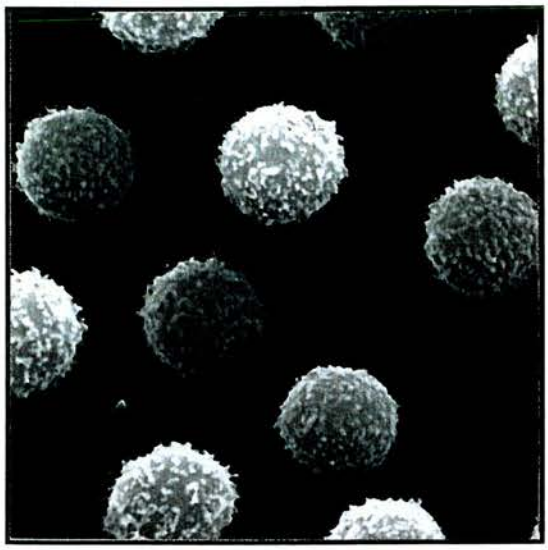
The enhanced responses following priming probably reflect both an increase in the response of individual cells and the recruitment of more cells to a responsive subpopulation (Bass *et al.*, 1986; Elbim *et al.*, 1993).

The induction of fMLP-induced shape change following priming was also of interest, not least because a very brisk polarization response is observed in human neutrophils and that is independent of priming status (Kitchen *et al.*, 1996b). In human neutrophils however, LPS exposure has been shown to enhance the rate and maximal extent, and reduce the lagtime, of F-actin polymerization induced by fMLP (Howard *et al.*, 1990). In equine neutrophils, the pre-incubation conditions necessary for LPS-induced priming of enhanced fMLP-stimulated Luci-DCL responses did not produce statistically significant cell shape change with LPS alone. Hence in this species, unlike in man (Haslett *et al.*, 1985), measurement of cell shape change alone, rather than more complex cytoskeletal changes, may not be the most appropriate or indeed accurate method of detecting cell priming or of modeling the control of cytoskeletal elements *in vitro*. This necessity for LPS priming to facilitate equine neutrophil shape change by functional fMLP receptor-ligand coupling could provide an

important and novel model to dissect signal transduction pathways linking priming responses to cell polarization.

Priming with PAF and TNF-α, however, did induce significant cell shape change in the absence of a respiratory burst, consistent with the human neutrophil priming scenario (Haslett *et al.*, 1985; Kitchen *et al.*, 1996b). The concepts of priming and subsequent activation of equine neutrophil effector functions derived from these data are offered as a summary paradigm in Figure 3.17.

Detailed characterization of the priming effect of LPS in equine cells demonstrated that enhancement of the fMLP-stimulated Luci-DCL response, although requiring slightly higher LPS concentrations (≥10 ng/ml) than human cells (Haslett *et al.*, 1985; Young *et al.*, 1990; Shapira *et al.*, 1995), evolved over a very similar time-course. In other species, the enhanced sensitivity of cells to LPS in the presence of serum (Aida and Pabst, 1990; Yang *et al.*, 1994; Shapira *et al.*, 1995) reflects the formation of a complex between LPS and a serum LBP, recognized by the glycosyl-phosphatidylinositol-anchored membrane protein, mCD14 (Wright *et al.*, 1990; Yang *et al.*, 1995). CD14 co-operates with the pattern recognition receptor, Toll-like receptor-2, to stimulate signal transduction and cell activation (Yang *et al.*, 1998). The ability of human and equine serum to substitute for one another in these experiments suggests that a functional homologue of LBP is indeed present in equine serum and provides strong evidence for a similar paradigm underlying at least one mechanism of LPS interaction with equine neutrophils. However, the presence of a mCD14 cannot be inferred directly from these data, as complexes of LPS and soluble (s)CD14 can stimulate human neutrophils with similar potency to LPS/LBP (Hailman *et al.*, 1996). However, it seems unlikely for an equine sCD14 to be present in plasma in the absence of a functional mCD14 homologue in neutrophils. LPS has also been demonstrated to activate human neutrophils in the absence of serum proteins (Ulevitch and Tobias, 1995), via L-selectin (Malhotra *et al.*, 1996), the integrin CD11c/CD18 (Ingalls and Golenbock, 1995) and both soluble (Hailman *et al.*, 1996) and membrane-bound CD14 (Shapira *et al.*, 1995). One or more of these pathways might be involved in the priming of equine neutrophils observed in the absence of serum. Although work is underway to define an equine CD14 (Dr J.N. Moore, personal communication), the existence of CD14, a more detailed



SEM of freshly isolated cells (x 11560)

Quiescent:

Cells spherical

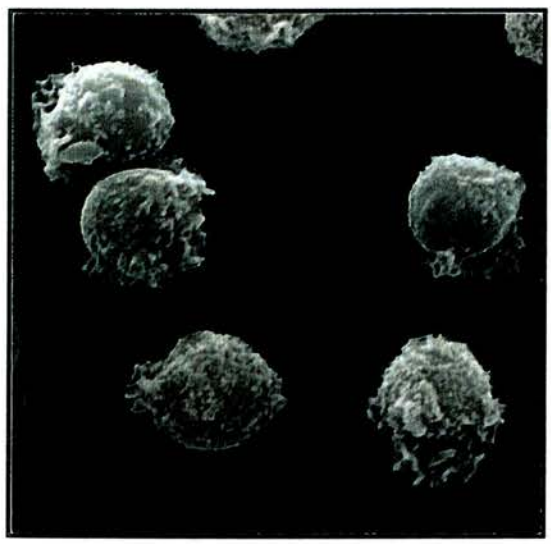
No respiratory burst

LPS

TNF-α

PAF

Potentially reversible both *in vitro* and *in vivo* ?



SEM of LPS + serum primed cells (x 12240)

Primed:

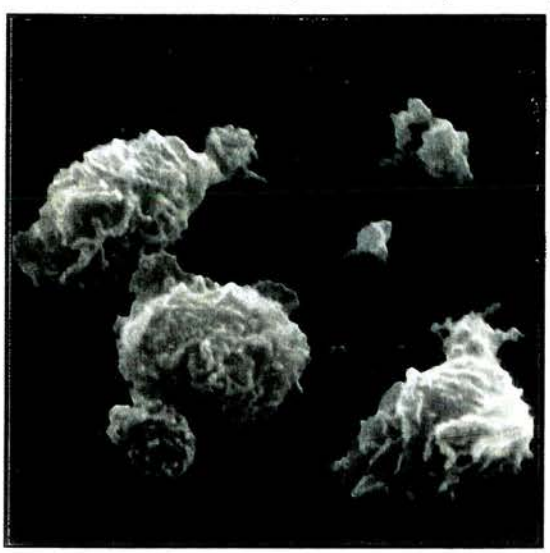
Surface ruffles, some pseudopodia

No respiratory burst

fMLP

ZAP

PMA ?



SEM of ZAP-stimulated cells (x 18500)

Activated:

Polarization

Large respiratory burst

fMLP receptor coupling

All other responses enhanced if primed

Figure 3.17: Proposed paradigm of equine neutrophil priming and activation

dissection of this potential LBP/CD14 mechanism and the elucidation of other LPS receptors in neutrophils, remain to be explored in this species.

The chemotaxis data presented (Figure 3.15) are in agreement with most previous reports in equine neutrophils (Snyderman and Pike, 1980; Camp and Leid, 1982; Naef *et al.*, 1982), which showed no chemotactic effect of fMLP. However, Zinkl and Brown (1982) and Sedgwick *et al.* (1987) observed a chemotactic effect of fMLP but only at extremely high, non-physiological, concentrations i.e. 100 μM. Taken together, these data suggest that fMLP is at best a very poor chemotactic agent for equine neutrophils and that LPS exposure is unable to modulate this lack of response. Of note, LPS exposure actually reduces the chemotactic responsiveness of human neutrophils to fMLP (Haslett *et al.*, 1985), but other priming agents (e.g. GM-CSF) can enhance fMLP-directed chemotaxis (Weisbart *et al.*, 1986); however the response of equine neutrophils to ZAP in our system was unaffected by LPS (data not shown).

Scatchard analysis of [^{3}H]fMLP binding to equine neutrophils was able to confirm the presence of specific fMLP binding sites in equine neutrophils and also identified a 2.5-fold increase in fMLP receptor number following LPS priming, without altering the single receptor site model fit or changing receptor affinity. A similar single site model for equine neutrophils was described by Snyderman and Pike (1980) in which they identified 630 ± 184 receptors per cell, equivalent to the number we found in unprimed cells. A single fMLP receptor site has also been identified in rabbit neutrophils where receptor expression was increased eight-fold following intravenous endotoxin administration but this was not accompanied by any comparable effect on neutrophil function, suggesting that the two effects were not directly related (Goldman *et al.*, 1986). This point is reinforced by studies in human neutrophils which have demonstrated that the increase in fMLP receptor expression observed in response to priming (Zimmerli *et al.*, 1990; O'Flaherty *et al.*, 1991) or bacterial infection (Follin *et al.*, 1989), either lags behind (O'Flaherty *et al.*, 1991) or does not correlate with (Follin *et al.*, 1989; Zimmerli *et al.*, 1990), the enhanced functional response. Increased receptor numbers result from translocation of preformed fMLP receptors from intracellular compartments to the plasma membrane as a consequence of fusion of specific granules and particularly secretory vesicles

with the cell surface during priming (Sengelov *et al.*, 1994). The probability that the small change in fMLP receptor number identified in equine cells post LPS treatment is a consequence, rather than the cause, of priming is supported by the observation that receptor numbers for other human neutrophil agonists (e.g. C5a), whose responses are equally enhanced, are not affected by LPS (Zimmerli *et al.*, 1990). It is unlikely therefore, that a change in receptor number alone represents a major control point determining the magnitude of the fMLP receptor-mediated response. Hence, the dramatic augmentation of the fMLP response observed, most likely occurs at a level downstream from ligand-receptor binding in the signal transduction cascade (Condliffe *et al.*, 1998b). Indeed many of the intracellular biochemical alterations linked to priming have been shown to be dissociated from primed effector functions and much work is in progress to differentiate the biochemical consequences of priming from its origins (Downey *et al.*, 1995; Condliffe *et al.*, 1998b). Further studies are required to dissect both the precise mechanism of LPS interaction with equine neutrophils and the subsequent intracellular signalling steps that stimulate functional coupling of fMLP receptors. However, this very dramatic and selective priming response observed in the equine neutrophil may offer an ideal model for the investigation of functional coupling of leukocyte G-protein-linked chemoattractant receptors.

Neutrophil priming has significant pathophysiological consequences *in vivo* and characterization of priming and activation in equine neutrophils *in vitro* was the initial step toward being able to evaluate the functional status of neutrophils harvested from the peripheral blood and airways of COPD-susceptible horses following hay/straw challenge. Indeed, the observation that priming appeared to be a prerequisite for functional coupling of fMLP receptors in equine neutrophils suggested that the *ex vivo* response of peripheral blood and airspace neutrophils to fMLP could be employed as an "index of functional priming".

In vivo priming of neutrophil oxidative activity has been observed experimentally following intravenous infusion of inflammatory mediators such as LPS (Cerasoli *et al.*, 1990) and TNF-α (Wewers *et al.*, 1990; Kapp *et al.*, 1991) and clinically in association with bacterial infection (Bass *et al.*, 1986; Follin *et al.*, 1989), severe trauma (Botha *et al.*, 1995), ARDS (Zimmerman *et al.*, 1983; Chollet-Martin *et al.*,

1992) and allergic asthma (Meltzer *et al.*, 1989) in man. Also intravenous administration of LPS to horses enhanced peripheral blood neutrophil oxidative activity measured by reduction of nitrotetrazolium blue (Krumrych *et al.*, 1997). However, many of these examples are likely to involve a complex interactive network of pro-inflammatory stimuli in plasma.

The primed state of human neutrophils exudating into suction-induced skin chambers (Zimmerli *et al.*, 1986; Follin *et al.*, 1991) and the observation that ligation of neutrophil β_2-integrins *in vitro* also primes these cells, demonstrates the interactions between adhesion and priming (reviewed in Section 1.4.1).

The pivotal role of priming in determining neutrophil behaviour is re-inforced by the recent recognition that the pro-inflammatory fate of primed cells is not immutable. Isolated neutrophils can undergo a dynamic cycle of priming - depriming and repriming (Kitchen *et al.*, 1996b) and although priming is stabilised by plasma (Brown *et al.*, 1997), this flexibility infers a subtle mechanism for the limitation of collateral host tissue damage at an inflamed site. Moreover, the profound, yet diverse, regulatory effects that priming agents can have upon the constitutive rate of neutrophil apoptosis, may strongly influence the manner in which an inflammatory responses resolves (Lee *et al.*, 1993; Murray *et al.*, 1997; Savill, 1997a).

CHAPTER 4

CHARACTERIZATION OF APOPTOSIS IN EQUINE PERIPHERAL BLOOD NEUTROPHILS *IN VITRO*

4.1 INTRODUCTION

The capacity of the neutrophil to accumulate at an inflamed site and to secrete pro-inflammatory and histotoxic agents ensures that this cell plays a key role in the initiation, amplification and/or resolution of acute inflammation. Indeed, neutrophils have been implicated as mediators of tissue destruction in a wide range of inflammatory diseases in both man and the horse (reviewed in Section 1.6).
The seminal work of Newman *et al.* (1982) demonstrating phagocytosis of "aged" neutrophils by macrophages initiated investigation of the behaviour of neutrophils as they aged in culture as a model for their fate following accumulation at an inflammatory site. A series of detailed studies by Haslett and colleagues demonstrated that neutrophils underwent constitutive apoptosis (reviewed in Section 1.5) when aged in culture and this was the signal for initiation of a novel macrophage recognition mechanism (Savill *et al.*, 1989a; Savill *et al.*, 1989b; Haslett *et al.*, 1989b; Savill *et al.*, 1990). Furthermore, demonstration that this paradigm was active *in vivo* (Savill *et al.*, 1989b) hinted at an explanation for the clearance of a large neutrophil burden without permanent structural tissue damage in the resolution of neutrophil-dependent diseases such as pneumococcal pneumonia in humans (Haslett, 1992). Downregulation of the functional integrity of neutrophils undergoing apoptosis (Lee *et al.*, 1993; Whyte *et al.*, 1993b) gave credence to this hypothesis and suggested that pharmacological manipulation of apoptosis may offer therapeutic potential in inflammatory diseases in which neutrophils and their histotoxic products had been implicated (Haslett, 1992). It was clear that if neutrophil apoptosis was to be harnessed to beneficial therapeutic effect, several questions required immediate attention. Firstly, how interaction with inflammatory mediators, other host tissue structures and pathogens might regulate neutrophil

longevity. Secondly, the intracellular signalling pathways and effector molecules regulating the apoptotic programme. Finally, what changes in neutrophil and macrophage phenotype might facilitate the recognition and clearance of effete neutrophils (Savill *et al.*, 1993; Haslett, 1997; Savill, 1997a).

The development, maturation and functional status of mammalian neutrophils are finely regulated by a broad array of growth factors, cytokines and bacterial products, to tailor cell number, functional capacity and longevity to the changing demands of host defence in everyday life (Condliffe *et al.*, 1998b). Following the observation that inflammatory mediators such as LPS, GM-CSF and C5a inhibited the rate of constitutive human neutrophil apoptosis and *pari passu* prolonged functional longevity (Lee *et al.*, 1993), a great number of inflammatory mediators and potential therapeutic compounds have been screened for their ability to modulate both apoptosis and the recognition of effete cells.

An initial paradigm suggesting that mediators with the capacity to prime or activate neutrophils would promote survival (with the concomitant risk of enhancing the potential for tissue destruction) is now recognized as an oversimplification of nature's inherent capacity to ensure the survival of the whole organism. The teleological sagacity of this innate regulation began to emerge with the identification of an early pro-apoptotic effect of the inflammatory cytokine TNF-α (Takeda *et al.*, 1993; Murray *et al.*, 1997). More recent studies have identified a similar modulatory role for several fundamental priming/activation-dependent functions (such as β_2-integrin engagement and transendothelial migration) in stimulating apoptosis as a direct result of neutrophil recruitment (Coxon *et al.*, 1996; Walzog *et al.*, 1997; Watson *et al.*, 1997b). Although there are discrepancies in the literature e.g. (Baran *et al.*, 1996), in general, phagocytosis of opsonised bacteria and other particles (Coxon *et al.*, 1996; Watson *et al.*, 1996a; Watson *et al.*, 1996b) appears to rapidly induce apoptosis leading to early removal of both the activated neutrophil and its pathogenic prey. The nature of the immune complexes involved in particle opsonisation and the mechanism of their subsequent recognition may further regulate this process by the neutrophil (Gamberale *et al.*, 1998).

Apoptosis within an inflammatory focus may be modulated by many physico-chemical signals. Heat shock (Watson *et al.*, 1996a) and alkaline conditions

(Leblebicioglu and Walters, 1999) drive apoptosis *in vitro*, whereas hypoxic conditions significantly prolong cell survival (Hannah *et al.*, 1995); all micro-environmental factors that may be profoundly perturbed in an inflamed nidus. My recognition of cell morphology consistent with the neutrophil apoptosis / macrophage phagocytosis paradigm in clinical cytology preparations of neutrophilic equine BAL, synovial, uterine and peritoneal fluids led to the current investigation and characterization of equine neutrophil apoptosis (T. Brazil, unpublished observations, 1995 - 1996).

4.2 RESULTS

4.2.1 CHARACTERIZATION OF APOPTOSIS IN EQUINE NEUTROPHILS

4.2.1.1 Equine neutrophils aged in culture demonstrate morphological features of apoptosis

In preliminary experiments, light microscopic examination of Diff Quik-stained cytocentrifuge preparations of isolated equine neutrophils aged in culture for 8 h, revealed a small proportion of cells exhibiting morphological changes consistent with apoptosis (Savill *et al.*, 1989b; Kerr *et al.*, 1995). The most prominent features were nuclear pyknosis with condensation of nuclear chromatin into one or more, densely staining, rounded remnants and cytoplasmic vacuolation (Figure 4.1c). These cells were readily distinguished from non-apoptotic neutrophils (Figures 4.1a & c). Ultrastructural examination of these cells revealed the stereotypical morphology of apoptosis (Kerr *et al.*, 1972; Savill *et al.*, 1989b; Kerr *et al.*, 1995). Nuclear chromatin was condensed into clearly demarcated, typically ovoid, fragments with margination of denser, more granular aggregates that frequently formed a crescent, tightly apposed to the inner surface of the nuclear envelope (Figure 4.2b). Cytoplasmic vacuolation was prominent and intracellular organelles, including cytoplasmic granules, although appearing crowded, were retained (Figure 4.2b). Moreover, despite loss of the small pseudopodia found in freshly isolated cells, the cell membrane remained intact (Figures 4.2a & b). Nucleolar prominence,

another common ultrastructural feature of apoptosis in other cell types (Savill *et al.*, 1989b; Kerr *et al.*, 1995), was infrequently observed, but this may just reflect the absence of nucleoli from the planes of the sections studied (Kerr *et al.*, 1995). Further quantitative TEM would be required to determine the true frequency of this ultrastructural feature. The intense plasma membrane ruffling, blebbing or budding [sometimes referred to as zeiosis (Majno and Joris, 1995)] that is associated with cytoskeletal modification during apoptotic cell death is well recognized in many cell types (Cohen *et al.*, 1992; Kerr *et al.*, 1995; Majno and Joris, 1995). This feature has not been routinely reported in apoptotic neutrophils but dramatic membrane blebbing has been observed in neutrophils driven to undergo apoptosis by the protein synthesis inhibitor, cycloheximide (Sullivan *et al.*, 1996). However, whilst counting apoptotic cells in a haemocytometer, very occasional equine neutrophils were seen to have such a bizarre, budding appearance. Such morphological changes are likely to be short-lived (Kerr *et al.*, 1995) and thus undetectable or overlooked without sequential scanning electron or phase contrast microscopic studies.

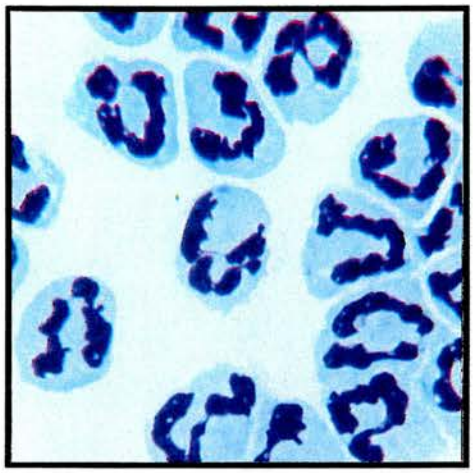


Figure 4.1a: Freshly isolated cells
Light microscopic morphology
(Diff-Quik, x 1100)

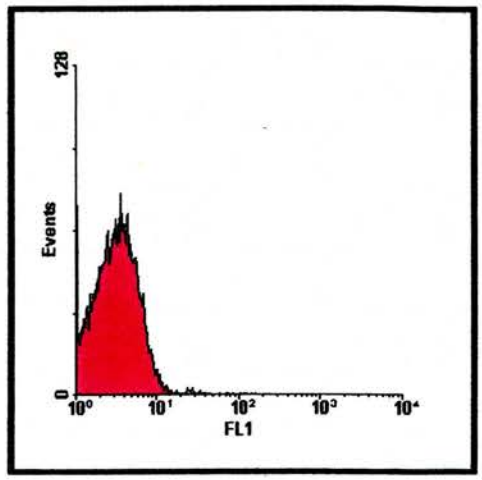


Figure 4.1b: Freshly isolated cells
Annexin V binding (10,000 events)

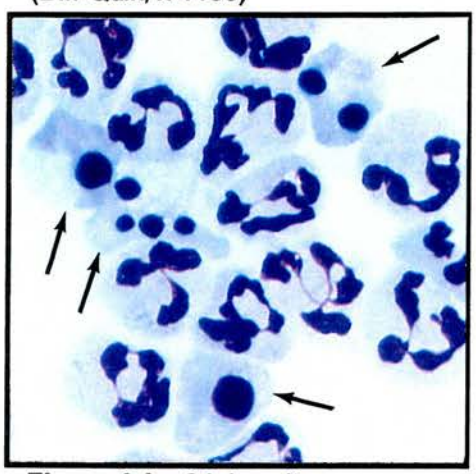


Figure 4.1c: 8 h in culture
Light microscopic morphology
(Diff-Quik, x 1100)

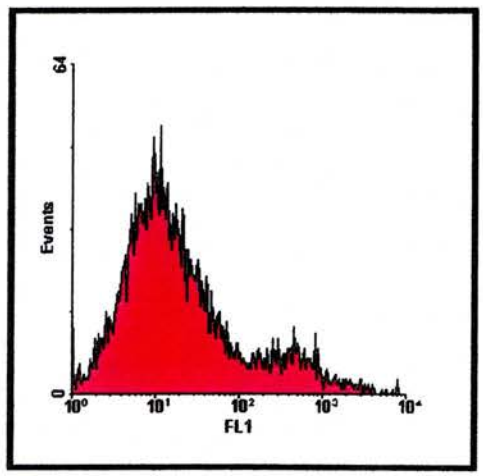


Figure 4.1d: 8 h in culture
Annexin V binding (10,000 events)

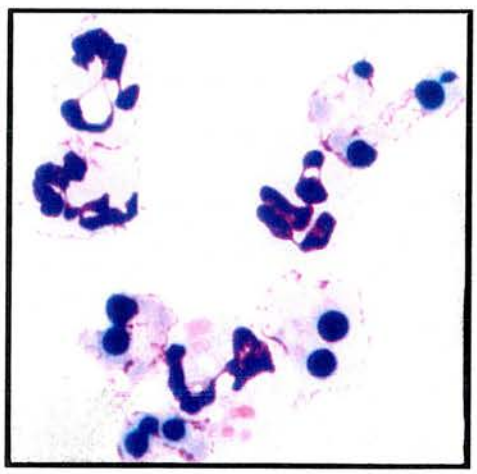


Figure 4.1e: 20 h in culture
Light microscopic morphology
(Diff-Quik, x 1100)

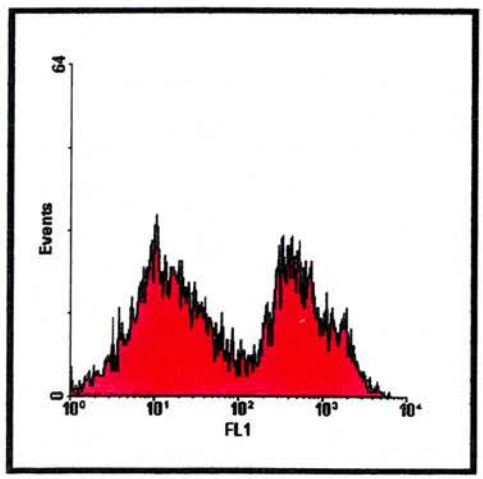


Figure 4.1f: 20 h in culture
Annexin V binding (10,000 events)

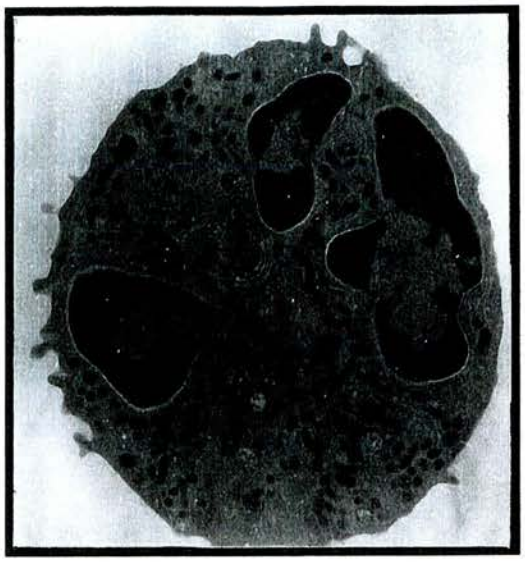


Figure 4.2a: Transmission electron photomicrograph of a freshly isolated equine neutrophil.

Cells prepared as described in Section 2.3.2. (Magnification x 13000)

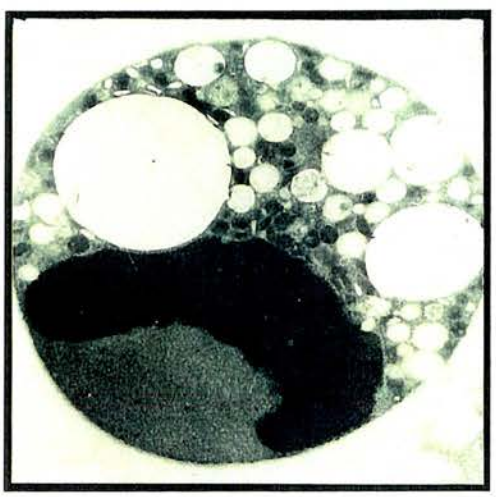


Figure 4.2b: Transmission electron photomicrograph of an equine neutrophil aged in culture for 20 h.

Cells prepared as described in Section 2.3.2. (Magnification x 12000)

4.2.1.2 Equine neutrophils undergo time-dependent constitutive apoptosis *in vitro*

Equine neutrophils aged in culture underwent time-dependent constitutive apoptosis with 6.7 ± 1.6% (mean ± SEM, n = 4), 27.7 ± 5.4%, 39.9 ± 3.2%, 53.6 ± 5.1%, 60.1 ± 6.3%, and 68.2 ± 3.3% of cells having typical light microscopic morphology at 6, 12, 18, 24, 30 and 36 h respectively (Figure 4.3). Membrane integrity and hence cell viability, was maintained throughout this time course with 99.8 ± 0.1% (mean ± SEM, n= 4), 99.6 ± 0.2%, 99.0 ± 0.4%, 98.8 ± 0.6%, 97.6 ± 0.4% and 97.7 ± 3.3% of cells continuing to exclude trypan blue at 6, 12, 18, 24, 30 and 36 h respectively (Figure 4.3). Moreover, cell loss was minimal (Figure 4.3), despite the presence of a small but increasing numbers of cell ghosts (anucleate cells) beyond 24 h. In all subsequent experiments, the effects of time and various interventions on the rate of apoptosis were quantified by light microscopy of cell populations harvested after 8 and 20 h in culture. The light microscopic morphology of cells harvested after 0, 8 and 20 h of culture is shown in Figures 4.1a, c and e.

4.2.1.3 Apoptosis in equine neutrophils is associated with the ability of cells to bind Annexin V

Freshly isolated equine neutrophils demonstrated minimal Annexin V binding (Figure 4.1b). As cells aged in culture, an increasing number acquired the ability to bind Annexin V; this effect paralleled the appearance of typical apoptotic morphology (Figure 4.1 a-f). This suggested that equine neutrophils undergoing apoptosis experience the typical loss of plasma membrane asymmetry, with exposure of phosphatidylserine on the external leaflet, as recognized in other species' cells, including neutrophils (Fadok *et al.*, 1992; Homburg *et al.*, 1995).

Other workers have suggested that structural re-organisation of plasma membrane phospholipids is an early event in the apoptotic cascade and may precede both the appearance of typical morphological changes and fragmentation of DNA (Mower *et al.*, 1994). The proportion of equine neutrophils binding Annexin V was strongly correlated with the proportion of apoptotic cells based on morphological criteria ($r = 0.92$, $p<0.0001$, pooled data after 0, 8 and 20 h in culture from 11 separate experiments each performed in triplicate (morphology) and duplicate (Annexin V

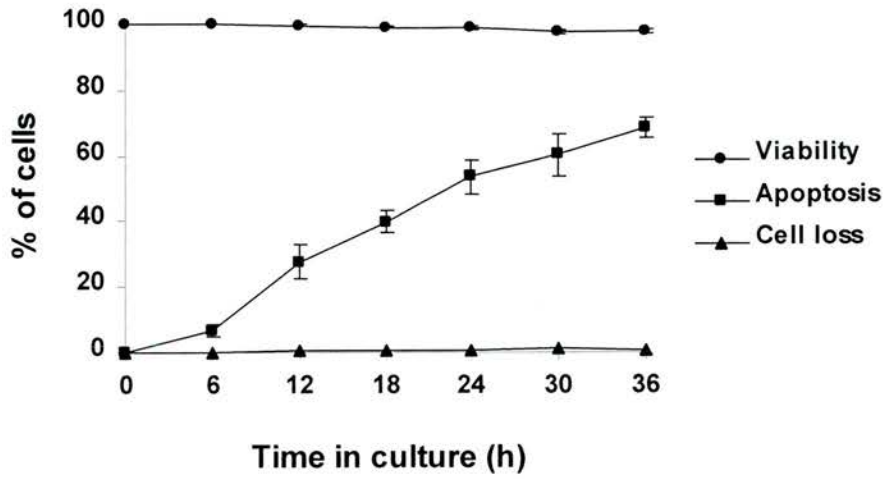


Figure 4.3: **Time course of constitutive apoptosis in equine neutrophils**
Equine neutrophils (5 x 10^6 cells/ml) were incubated as described in Section 2.6.1and harvested at the time points indicated. Cells were resuspended and apoptosis (%) (squares) assessed morphologically on Diff Quik-stained cytocentrifuge preparations. Cell loss (triangles) and viability (circles) were determined from haemocytometer counts and the proportion of cells excluding trypan blue, respectively. Values represent mean ± SEM of four separate experiments each performed in triplicate (apoptosis) or duplicate (viability and cell loss).

binding), (raw data not shown). A regression line plotted from these data passed almost exactly through the origin, offering a statistical inference that these two features of constitutive equine neutrophil apoptosis are temporally associated (data not shown).

4.2.1.4 Exhibition of apoptotic morphology is associated with time-dependent internucleosomal DNA fragmentation

Data from numerous *in vitro* and *in vivo* cell culture systems suggest that DNA integrity and cell morphology should be examined in parallel to confirm that cell death has occurred via apoptosis (Bortner *et al.*, 1995). Although both single strand breaks and cleavage into large fragments are recognized in association with apoptosis in some cell types (Bortner *et al.*, 1995; Hale *et al.*, 1996), in general internucleosomal DNA cleavage produces the characteristic "DNA ladder"; the biochemical hallmark of the apoptotic process (Wyllie, 1980a; Bortner *et al.*, 1995; Squier *et al.*, 1995). Electrophoresis of DNA extracted from freshly isolated equine neutrophils demonstrated homogeneous, high molecular weight DNA with minimal electrophoretic mobility (Figure 4.4). After 8 h in culture, a classical "ladder" pattern of oligonucleosomal DNA fragments was observed and this was greatly enhanced after 20 h (Figure 4.4). DNA fragmentation assays are, at best, semi-quantitative, but work in murine thymocytes indicates that the quantity of lower molecular weight DNA present is related to the number of dying cells, indicating that apoptotic cells degrade all of their DNA (Wyllie and Morris, 1982).
Thus, qualitatively, these DNA fragmentation data are temporally comparable to the progression of neutrophil apoptosis quantified morphologically.

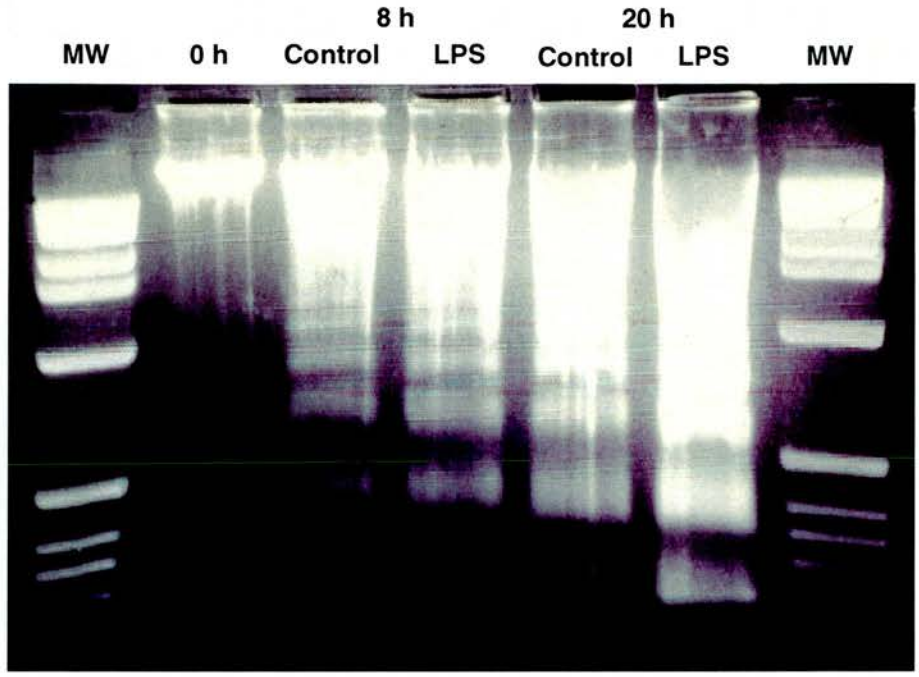


Figure 4.4: **DNA fragmentation in ageing equine neutrophils**

Electrophoresis in 1.6% agarose of DNA extracted as described in Section 2.6.4, from freshly isolated equine neutrophils (0 h) and cells incubated for 8 and 20 h in MF at 37°C either in the absence (Control) or presence of LPS (1 μg/ml). Ethidium bromide-stained DNA viewed by ultraviolet fluorescence. Note ladder pattern of low molecular weight DNA at 8 and 20 h time points that was enhanced in the presence of LPS; loss of high molecular weight DNA is especially prominent at 20 h in the presence of LPS. Experiment representative of three that gave similar results. (MW: DNA molecular weight markers; 1 kilobase ladder).

4.2.1.5 Simultaneous *in situ* identification of chromatin condensation and DNA fragmentation

The morphology and DNA integrity of individual cells can be assessed simultaneously under light microscopy by *in situ* staining of DNA by the TUNEL (Terminal deoxynucleotide transferase mediated d-UTP [desoxyuridinetriphoshphate]-biotin nick end-labelling) method (Ben-Sasson *et al.*, 1995). The TUNEL method can label breaks in DNA in individual nuclei *in situ* by the specific binding of the DNA polymerase, terminal deoxynucleotide transferase (TdT), to the exposed 3'-OH ends of cleaved DNA. The TdT catalyses the addition of biotinylated deoxynucleotides at this site. These are subsequently detected using a streptavidin-horseradish peroxidase conjugate. TUNEL staining of cytocentrifuge preparations of cells aged in culture for 8 h clearly localised fragmented DNA to cells with morphologically pycnotic nuclei and demonstrated the variation in the number and size of condensed chromatin fragments (Figure 4.5a & b). Negative control specimens (substitution of distilled water for TdT) showed no labelling and positive control specimens (pre-incubation of freshly isolated cell preparations with 1 μg/ml DNase 1 for 20 min after proteinase K exposure of DNA) demonstrated wide spread low grade labelling of cells that had normal nuclear morphology (data not shown).

Although TUNEL-positive cells were readily identified amongst non-apoptotic cells after 8 h in culture, poor resolution of individual TUNEL positive cells and a high level of non-specific labelling was observed in cells incubated for longer periods (e.g. 20 h) making accurate enumeration impossible. The TUNEL method is notorious for problems with sensitivity and particularly specificity, due to difficulties in obtaining homogenous exposure of DNA within condensed chromatin and the frequency of both physiological (both cell necrosis and proliferation can cause false positive TUNEL results) and preparation-related artefactual DNA breaks (Lucassen *et al.*, 1995; Labat-Moleur *et al.*, 1998). Hence, even with careful assessment of cell morphology, this technique was considered to be only of value for the qualitative identification of apoptotic chromatin in neutrophils cultured for short time periods.

Figure 4.5a: Photomicrograph of TUNEL-stained apoptotic equine neutrophils aged in culture for 8 h.

Cells aged in culture for 8 h were stained by TUNEL as described in Section 2.6.6. TUNEL positive nuclei were detected with DAB (brown staining) and sections counterstained with methyl green. (Magnification x 1200).

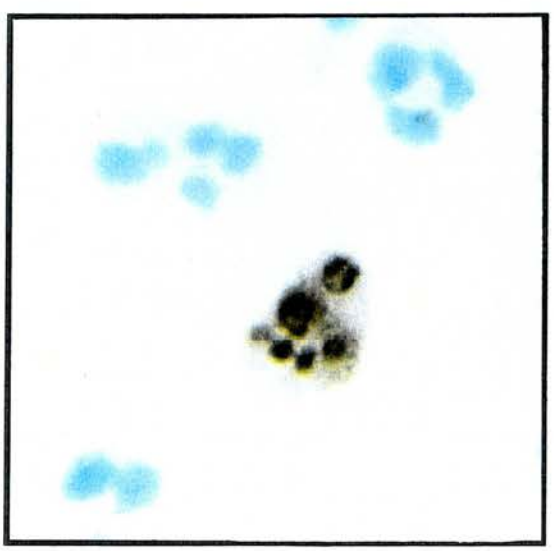


Figure 4.5b: Photomicrograph of TUNEL-stained apoptotic neutrophil aged in culture for 8 h demonstrating multiple condensed nuclear chromatin fragments.

Cells aged in culture for 8 h were stained by TUNEL as described in Section 2.6.6. TUNEL positive DNA detected with DAB (brown staining) and section counterstained with methyl green. (Magnification x 1380).

Although methods to improve the sensitivity and specificity of TUNEL in paraffin-embedded tissue sections have been suggested (Lucassen *et al.*, 1995; Labat-Moleur *et al.*, 1998), the reliability of the previous techniques precluded the need to pursue this method in cultured neutrophils.

In conclusion, light microscopic and ultrastructural morphology, exposure of Annexin V binding sites and evidence of internucleosomal DNA fragmentation confirmed that equine neutrophils undergo constitutive apoptosis *in vitro*.

4.2.1.6 Apoptosis is associated with down-regulation of receptor-mediated respiratory burst activity

Freshly isolated neutrophils mount a major respiratory burst in response to ZAP and PMA (Section 3.2.1). To investigate whether this capacity was maintained as cells aged and underwent apoptosis and whether the different mechanisms of cellular activation (receptor-mediated versus direct activation of protein kinase C) were important, Luci-DCL in response to these secretagogue agonists was measured before and after 20 h in culture. After 20 h, ZAP-stimulated Luci-DCL was significantly diminished to 19.6 ± 12.2% (n = 3) of the baseline response, in spite of only 50.3 ± 11.6% of cells having morphological features of apoptosis (Figure 4.6). This was not due to any reduction in cell viability as > 99% of cells continued to exclude trypan blue. This suggested that the diminished CL response of ageing cells preceded visible chromatin condensation. Further evidence that apoptotic cells remained viable was provided by the preservation of the PMA-stimulated response (107.4 ± 32.3% of baseline) indicating that the cells' capacity to assemble their NADPH oxidase and generate a respiratory burst remained intact. These data give provisional evidence for equine neutrophils becoming insulated from physiological receptor-mediated secretagogue stimuli at an early stage after commitment to apoptotic death, effectively disabling their pro-inflammatory potential. This hypothesis could be pursued by studying the response to these and other receptor-mediated stimuli over a more detailed time course and investigating receptor expression where appropriate. Ideally, populations of apoptotic and non-apoptotic cells should be purified, to test whether the CL response of non-apoptotic cells after 20 h is equivalent to that measured immediately following isolation. This separation

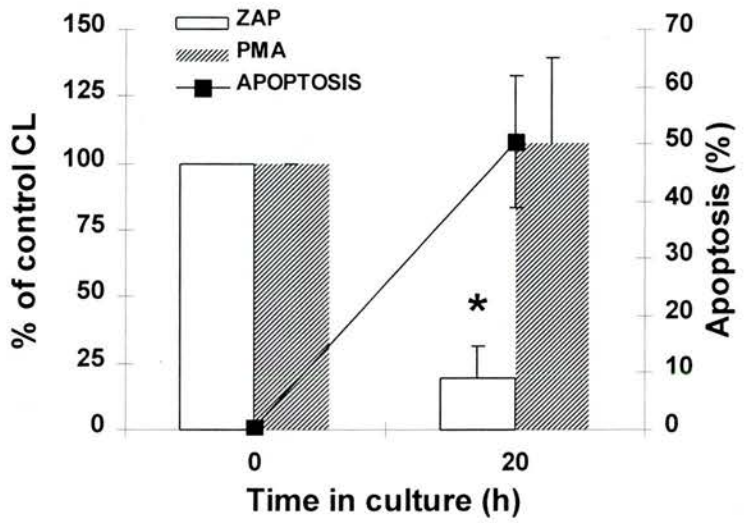


Figure 4.6: Chemiluminescence response of equine neutrophils undergoing apoptosis
ZAP (10% v/v)- and PMA (100 ng/ml)-stimulated Luci-DCL was measured in freshly isolated neutrophils and in cells harvested after 20 h in culture (ZAP: open bars, PMA: hatched bars). Apoptosis (closed squares) was assessed morphologically on Diff-Quik-stained cytocentrifuge preparations. Chemiluminescence (CL) data represents % of ZAP- and PMA-stimulated Luci-DCL in freshly isolated cells. Values represent mean ± SEM of three separate experiments each performed in triplicate. (* ; $p<0.05$)

might be achieved by counter-flow centrifugation with an elutriation rotor (Savill *et al.*, 1989b) or by negative selection of cells that did not bind Annexin V in a fluorescence-activated cell sorter (Homburg *et al.*, 1995).

4.2.2 REGULATION OF APOPTOSIS IN EQUINE NEUTROPHILS

Lee and colleagues (1993) demonstrated the ability of inflammatory mediators such as LPS, GM-CSF and C5a to inhibit neutrophil apoptosis *in vitro*, suggesting that the lifespan of neutrophils at an inflammatory site is likely to be markedly influenced by the complex array of mediators in the surrounding milieu.

In preliminary experiments, a number of priming and activating agents whose effects on equine neutrophils had already been characterized (see Chapter 3) were screened for their ability to regulate the rate of constitutive apoptosis in these cells. Apoptosis was routinely assessed in neutrophils after 8 and 20 h in culture to identify any temporal differences in the effect of a particular mediator. For some of these agents, human cells were examined in parallel in order to highlight any potential inter-species variation in the regulation of neutrophil apoptosis *in vitro*. Unfortunately, difficulties in co-ordinating the contemporaneous availability of human and equine cells in two different laboratories precluded undertaking such studies on a regular basis. Parallel studies were only performed if the cells were available for culture within 30 min of each other.

The bacterial product fMLP and the bioactive lipid PAF, had no effect on the constitutive rates of equine or human neutrophil apoptosis (Figure 4.7a & b). The eicosanoid, leukotriene B_4 (100 nM) although reported to inhibit human neutrophil apoptosis (Hebert *et al.*, 1996) had no effect on equine neutrophil apoptosis (data not shown). The human recombinant cytokine GM-CSF, a classical inhibitor of human neutrophil apoptosis (Lee *et al.*, 1993; Hebert *et al.*, 1996), again had no effect on the rate of equine neutrophil apoptosis but did significantly inhibit apoptosis in human cells after both 8 and 20 h in culture (% apoptosis: 8 h; Control 10.0 ± 1.2%, GM-CSF 2.9 ± 0.5%, $p<0.05$. 20 h; Control 50.1 ± 8.2%, GM-CSF 34.7 ± 7.4%, $p<0.05$, $n = 3$), see Figure 4.7a & b.

The most intriguing data in these preliminary studies were the consistent pro-

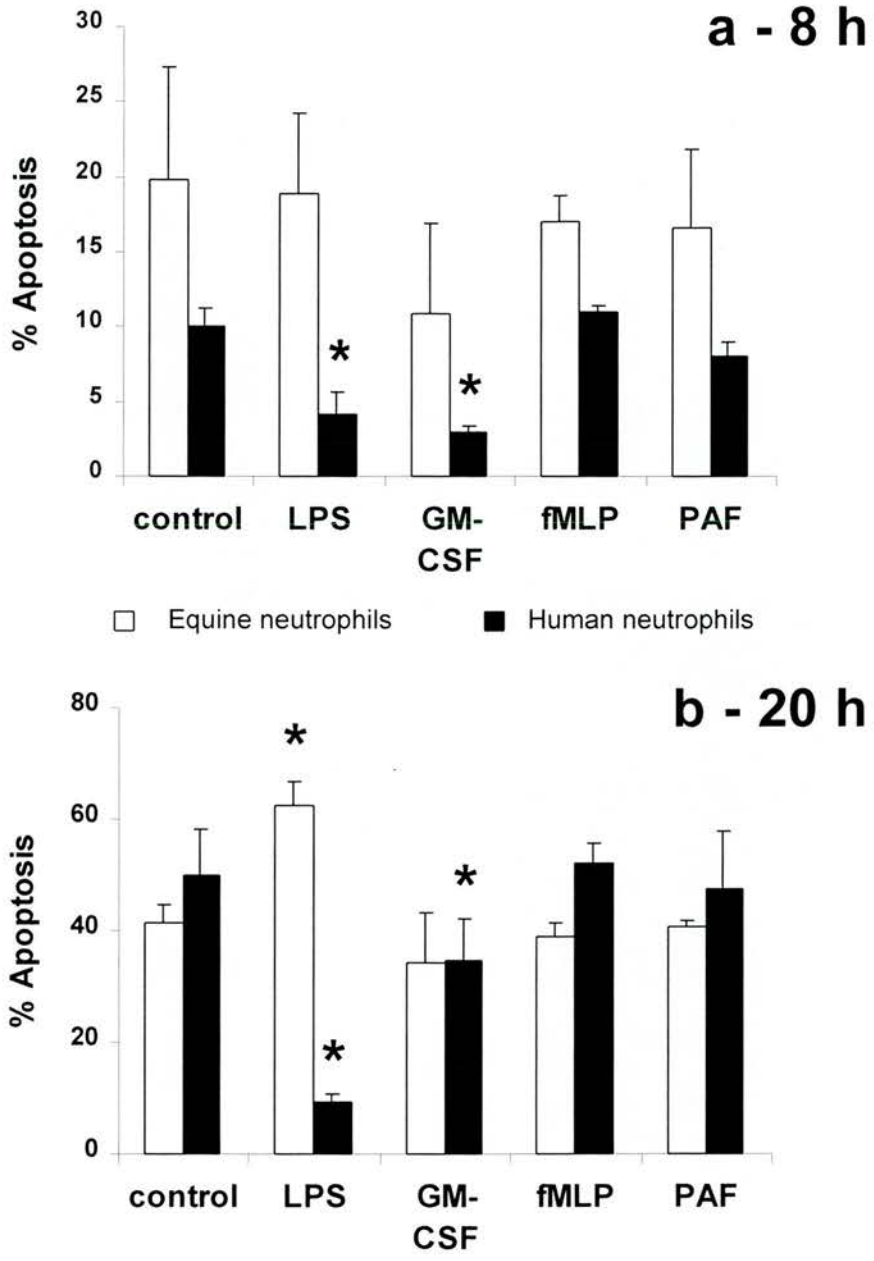


Figure 4.7a,b: Regulation of neutrophil apoptosis by inflammatory mediators; comparison of equine and human cells

Equine (open bars) and human (closed bars) neutrophils (5 x 10^6/ml) were incubated in MF at 37°C either alone (control) or in the presence of LPS (1 μg/ml), GM-CSF (100 U/ml), fMLP (1 μM) or PAF (1 μM) for (**a**) 8 or (**b**) 20 h. Cells were resuspended and percent apoptosis assessed morphologically on Diff Quik-stained cytocentrifuge preparations. Values represent mean ± SEM of three separate experiments, each performed in triplicate. (*; $p<0.05$ compared with time-matched species control).

apoptotic effect of LPS in equine cells at 20 h which contrasted strikingly with the marked inhibitory effect of LPS on human neutrophil apoptosis at both time points (Figure 4.7a & b). However, DNA fragmentation studies clearly showed enhanced chromatin cleavage in equine neutrophils in the presence of LPS at both 8 and 20 h (Figure 4.4). The detailed investigation of this effect is described below (Section 4.2.2.2).

4.2.2.1 ZAS inhibits equine neutrophil apoptosis

Equine neutrophils were incubated with 10% ZAS, as a biological source of C5a, to investigate the effect on the rate of apoptosis of a physiological ligand that directly triggers the respiratory burst. Studies in human neutrophils have reported both pro- (Hebert *et al.*, 1996) and anti-apoptotic (Lee *et al.*, 1993) effects of the complement fragment, C5a. ZAS was used because in initial experiments ZAP led to marked cell clumping at 20 h that made assessment of cytospins very difficult. Three experiments, utilizing different donors, compared the abilities of ZAP and ZAS (prepared from the same blood sample) to stimulate a respiratory burst. These produced almost identical results (data not shown). ZAS (10% v/v) profoundly inhibited equine neutrophil apoptosis at both 8 and 20 h (Figure 4.8) suggesting that activation of neutrophils at an inflammatory site has the potential to significantly prolong their lifespan.

To test this hypothesis further, equine cells were incubated with PMA (0.1 – 1000 ng/ml), another soluble agonist with the ability to directly trigger the respiratory burst. At concentrations less than 10 ng/ml no effect on the rate of constitutive apoptosis was observed. However, at concentrations greater than 10 ng/ml, there was significant loss of both cell numbers and viability (trypan blue exclusion) after only 8 h in culture (10 ng/ml PMA: 70% recovery and 93% viability, mean of n = 2). Moreover, after both 8 and 20 h, this concentration of PMA resulted in an increased number of cells with abnormal morphology (% normal morphology: 8 h; control 90.2, PMA 45.8. 20 h; control 59.1, PMA 31.1, mean of n = 2 experiments). The abnormal cells were typically swollen and highly vacuolated; some had enlarged nuclear lobes connected by fine strands of chromatin, others had loosely and irregularly fragmented nuclear material suggestive of nuclear rupture. Of note,

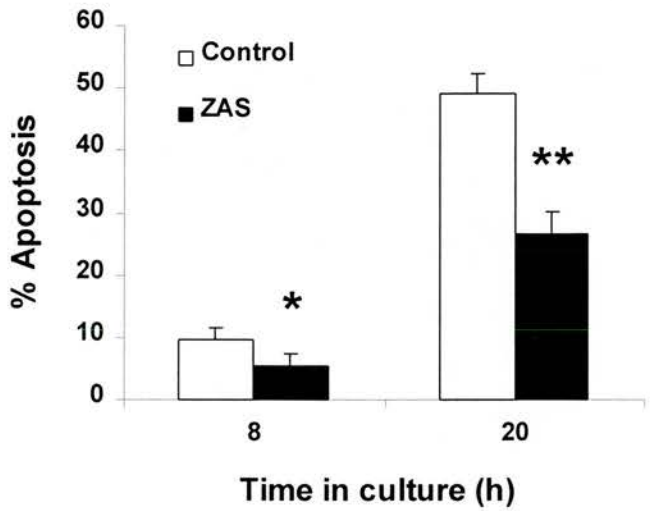


Figure 4.8: ZAS, a biological source of C5a, inhibits equine neutrophil apoptosis *in vitro*

Equine neutrophils (5 x 10^6/ml) were incubated in MF at 37°C either alone (open bars) or in the presence of ZAS - 10% v/v (closed bars) for 8 and 20 h. Cells were resuspended and percent apoptosis assessed morphologically on Diff-Quik-stained cytocentrifuge preparations. Values represent mean ± SEM of six separate experiments, each performed in triplicate. (* ; $p<0.05$, ** ; $p<0.001$ compared with time-matched control).

Raidal *et al.* (1998a) reported significant cell lysis following a 30 min incubation of equine neutrophils with 100 ng/ml PMA. These bizarre morphological features observed in response to PMA have also been described in human neutrophils with both PMA and following ingestion of opsonized zymosan and most likely represent a form of toxic or oxidative stress-induced death that is different to both classical apoptosis and necrosis (Takei *et al.*, 1996).

4.2.2.2 LPS promotes apoptosis in equine neutrophils

Having identified an unexpected pro-apoptotic effect of LPS in equine neutrophils, further studies were performed to confirm and dissect this effect in more detail. In a series of ten experiments, LPS (1 μg/ml) consistently enhanced apoptosis at both 8 and 20 h (Figure 4.9a). This pro-apoptotic effect was both time and concentration-dependent (8h; EC_{50} 96.7 ± 2.5 ng/ml, 20h; EC_{50} 32.3 ± 2.4 ng/ml, n = 3, see Figure 4.9b). Moreover, this was confirmed by DNA fragmentation studies at both 8 and 20 h (Figure 4.4).

The marked contrast to the data acquired in parallel from human cells (Figure 4.7a & b) suggested that this effect was a novel variation in equine neutrophils. Further experiments were performed to discount a number of potentially artefactual causes for this finding.

To ensure that that this effect was not specific to a particular batch or type of LPS, equine and human cells were incubated in parallel with two different batches of *Escherischia coli* O111.B4 (the LPS type used routinely in these studies) and a rough mutant *Salmonella typhimurium* LPS Ra 60. At 20 h, all three forms of LPS consistently promoted apoptosis in equine neutrophils and inhibited apoptosis in human neutrophils (data not shown).

LPS is well recognized to upregulate the expression of leucocyte integrins (Bochsler *et al.*, 1990) and to stimulate neutrophil adhesion to a variety of surfaces (Young *et al.*, 1990; Worthen *et al.*, 1992) and this effect is enhanced in the presence of plasma (Hailman *et al.*, 1996). We hypothesised that any enhancement of LPS-stimulated adhesion of non-apoptotic equine neutrophils to the flexiwells in comparison to human cells, could lead to a lower recovery of non-apoptotic cells, thereby artificially increasing the proportion of apoptotic equine cells harvested. This could

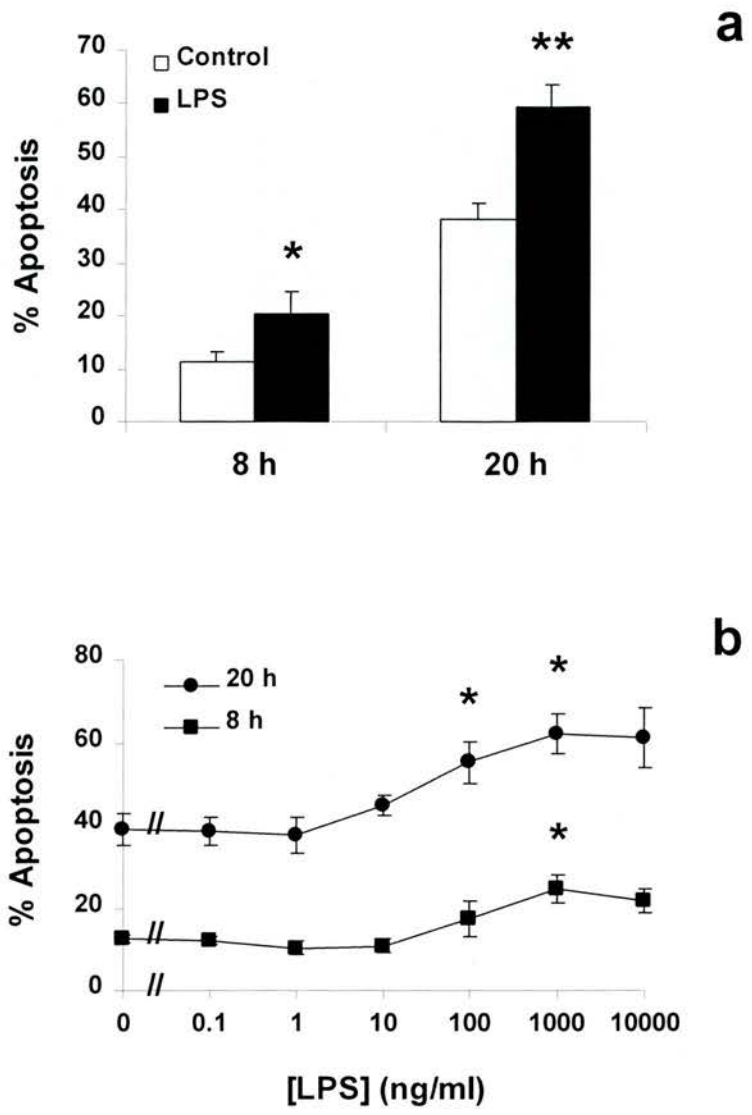


Figure 4.9a,b: **LPS enhances apoptosis in equine neutrophils *in vitro***

Equine neutrophils (5 x 10^6/ml) were incubated in MF at 37°C for 8 and 20 h. Cells were resuspended and percent apoptosis assessed morphologically on Diff Quik-stained cytocentrifuge preparations.

a: Cells incubated in the absence (open bars) or presence of 1 μg/ml LPS (closed bars). Values represent mean ± SEM of ten separate experiments, each performed in triplicate.

b: Cells incubated, either alone or in the presence of LPS (0.1 - 10000 ng/ml) for 8 h (squares) and 20 h (circles). Values represent mean ± SEM of three separate experiments, each performed in triplicate.

(* ; $p<0.05$, ** ; $p<0.001$ compared with time-matched control).

lead to the erroneous conclusion that LPS was pro-apoptotic in equine neutrophils. Therefore, an experiment was performed comparing equine and human neutrophil adhesion and apoptosis, in parallel. Cells were harvested using the standard resuspension technique (Section 2.6.2) and the proportion of cells that remained adherent to the wells was quantified by methylene blue staining, subsequent cell lysis and measurement of optical density (Section 2.6.2.1). Although adhesion was slightly enhanced in the presence of LPS, this effect was greater in human than equine cells and the same pro- and anti-apoptotic responses were observed (20 h data is shown in Figure 4.10), effectively ruling out an adhesion-related effect.

4.2.2.3 Effect of TNF-α on apoptosis in equine neutrophils

While TNF-α has been demonstrated to induce apoptosis in human neutrophils, most studies suggest that this is a transient (2 – 12 h) effect (Takeda *et al.*, 1993; Murray *et al.*, 1997), with TNF-α causing a paradoxical inhibition in the rate of apoptosis at later time points (Colotta *et al.*, 1992; Murray *et al.*, 1997). However, Watson and co-workers (1996a) reported a pro-apoptotic effect of TNF-α over all time points throughout a 25 h incubation period. Stimulated neutrophils are able to synthesise and release various cytokines including TNF-α (Cassatella, 1995) and LPS is a potent stimulus for TNF-α production (Dubravec *et al.*, 1990; Bazzoni *et al.*, 1991). This raised the possibility that the pro-apoptotic effect of LPS in equine neutrophils might be a paracrine response, secondary to the release of endogenous TNF-α. Therefore, the effect of human and erTNF-α on the rate of constitutive apoptosis in equine neutrophils and the effect of an equine TNF-α neutralising antibody on the pro-apoptotic LPS response were investigated.

Despite using the same batch of cytokine and taking care to maintain the standard protocol of cell culture and harvest throughout this series of experiments, the effect of hrTNF-α on equine cells was somewhat variable. The same batch of hrTNF-α produced a consistent pro-apoptotic effect in human neutrophils at 12.5 ng/ml (Dr. J. Murray, personal communication, data not shown). In preliminary experiments to determine the time dependence of any effect, apoptosis was assessed in cells incubated in the presence and absence of 25 ng/ml hrTNF-α for 2, 4, 6, 8, 10, 12 and 20 h. No statistically significant effect was observed at any time point. These

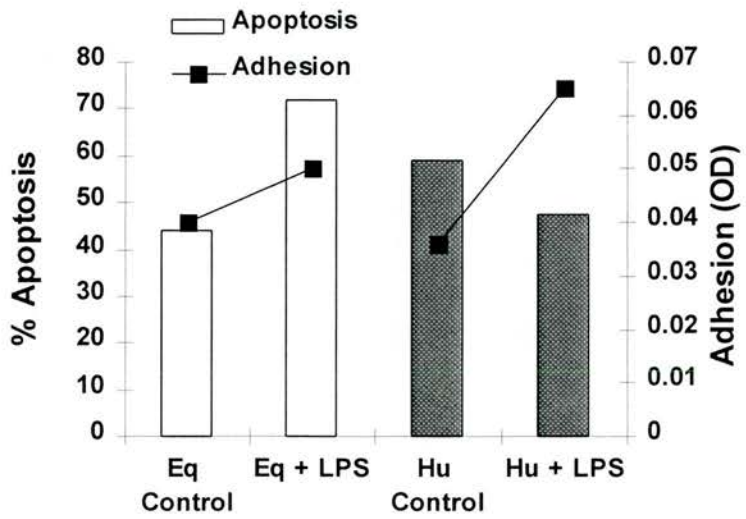


Figure 4.10: **The pro-apoptotic effect of LPS in equine neutrophils is unrelated to adhesion**

Equine (Eq - open bars) and human (Hu - hatched bars) neutrophils (5 x 10^6/ml) were incubated in MF at 37°C for 20 h, either alone or in the presence of 1 μg/ml LPS, as indicated. Cells were resuspended and percent apoptosis (bars: left-hand axis) assessed morphologically on Diff Quik-stained cytocentrifuge preparations. Following aspiration of entire well contents, adhesion (closed squares; right-hand axis) was assessed by methylene blue staining of cells, followed by cell lysis and measurement of optical density. Values represent the mean of triplicate determinations from a single experiment.

findings contrast with the highly consistent priming effect of this recombinant human cytokine in equine cells (Section 3.3.2.2).

In studies examining the effect of erTNF-α, the maximum final concentration of the cytokine available for use was only 1 ng/ml; this concentration however, potently primed equine cells (Section 3.2.3.2). Equine rTNF-α caused a small but significant enhancement of apoptosis at 8 h (% apoptosis; control 11.2 ± 2.2, erTNF-α 16.6 ± 3.6, $p<0.05$, n = 11). There was also a trend toward enhancement of apoptosis at 20 h, but this was not significant (% apoptosis; control 43.0 ± 3.1, erTNF-α 51.3 ± 4.2, $p>0.05$, n = 11). However, in 4 of these 11 experiments erTNF-α had either a minimal effect (3 experiments) or inhibited apoptosis (1 experiment). If these data were omitted from the analysis, a highly significant pro-apoptotic effect was observed at both time points in the remaining 7 experiments (% apoptosis: 8 h; control 10.6 ± 2.6, erTNF-α 20.3 ± 4.6, $p<0.001$; 20 h; control 40.6 ± 2.9, erTNF-α 57.4 ± 2.7, $p<0.01$, n = 7). This degree of donor variation in the response to erTNF-α made it impossible to draw firm general conclusions regarding the effect of TNF-α in neutrophils isolated from a random population of horses. However, variations in both TNF-α synthesis (McGuire *et al.*, 1994) and the sensitivity of neutrophils to the pro-apoptotic effect of TNF-α (Sendo *et al.*, 1996, Dr. J. Murray, personal communication) are well recognized within groups of human subjects and such heterogeneity in TNF-α responsiveness may be one explanation for the donor variation observed in these equine data.

When the TNF-α neutralising Mab was used to address the potential role of TNF-α in the pro-apoptotic effect of LPS in equine neutrophils, neither the neutralising Mab itself nor an isotype-matched control Mab affected either basal or LPS-induced apoptosis after 20 h (Figure 4.11b). The same antibody completely abrogated (91.0 ± 2.5%, n = 3) the priming effect of erTNF-α on fMLP-stimulated CL in equine neutrophils (Figure 4.11a), confirming its functional capability. An isotype-matched control Mab had no significant effect (Figure 4.11a).

This suggested that the novel pro-apoptotic effect of LPS in equine neutrophils was independent of neutrophil TNF-α generation.

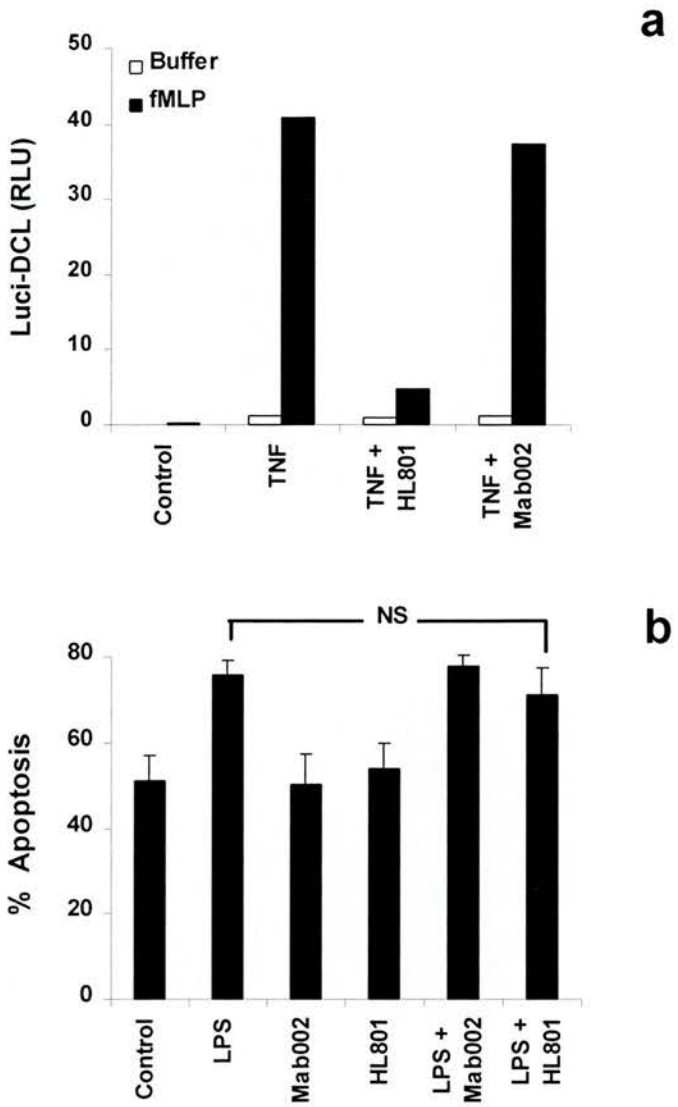


Figure 4.11a, b: **Effect of a TNF-α neutralising antibody on erTNF-α priming and LPS-induced apoptosis in equine neutrophils**

a: Cells were incubated with buffer (Control) or erTNF-α (1 ng/ml) in the presence and absence of either an equine TNF-α neutralising Mab (HL801, 1μg/ml), or an isotype control Mab (Mab002, 1 μg/ml) for 30 min prior to stimulation with buffer (open bars) or fMLP (closed bars, 1 μM). Values represent mean of triplicate determinations from a single experiment, representative of 3 that gave similar results. Pre-incubation with antibodies alone gave responses no different to control values.

b: Cells (5 x 10^6/ml) were incubated in MF at 37°C for 20 h, either alone or in the presence of LPS, HL801 (1 μg/ml) or Mab002 (1 μg/ml) or co-incubated with LPS and each Mab. Apoptosis was assessed morphologically. Values represent mean ± SEM of three separate experiments, each performed in triplicate. NS: not significantly different

4.2.2.4 Effect of phagocytosis on apoptosis in equine neutrophils

One of the primary roles of the neutrophil in the innate immune response is the phagocytosis and subsequent intracellular killing of invading micro-organisms. This process is greatly enhanced following opsonisation of micro-organisms by immunoglobulins or complement (Edwards, 1994a).

Ingestion of opsonised Gram negative bacteria (*Escherischia coli*) (Watson *et al.*, 1996a; Watson *et al.*, 1996b), Oil-red-O particles (Coxon *et al.*, 1996) and mouse erythrocytes (Gamberale *et al.*, 1998) stimulates apoptosis in human neutrophils. These observations are consistent with a teleological view that would predict direction of the phagocytosis-activated neutrophil toward non-phlogistic disposal. One of the most striking observations in the current study was the extent to which equine neutrophils were stimulated to undergo apoptosis rapidly following incubation with opsonised sheep erythrocytes. The basal rate of apoptosis was unaffected by incubation with non-opsonised cells (Figure 4.12). By 8 h, 74.7 ± 6.7% (morphological assessment, n = 6) of cells were apoptotic, compared to only 17.7 ± 2.6% of control cells and 17.4 ± 2.8% of cells incubated with non-opsonised erythrocytes (Figure 4.12a). In initial experiments, this pro-apoptotic effect in the presence of opsonised sheep erythrocytes was so dramatic that on some cytospin preparations it was difficult to accurately distinguish all the individual apoptotic cells. However, maintenance of the cells' ability to exclude trypan blue confirmed their viability.

To confirm these data, apoptosis was subsequently assessed by Annexin V binding in parallel with morphology (Figure 4.12). This indicated that morphological assessment appeared to identify a larger proportion of apoptotic cells. After 8 h in the presence of opsonised sheep erythrocytes, Annexin V binding identified only 57.5 ± 8.9% (n = 6) of cells as apoptotic. A similar enhancement of apoptosis in the presence of opsonised sheep erythrocytes was recognized morphologically at 20 h (% apoptosis; Control 44.6 ± 4.5%, OsRBC 93.3 ± 3.3, $p<0.01$, n = 6, Figure 4.12 c) and again Annexin V binding identified a smaller population of apoptotic cells (% apoptosis; Control 44.6 ± 3.9%, OsRBC 77.6 ± 4.2, $p<0.01$, n = 6, Figure 4.12d). Whether this discrepancy reflected difficulty in distinguishing individual apoptotic cells morphologically or some difference in the expression of Annexin V binding

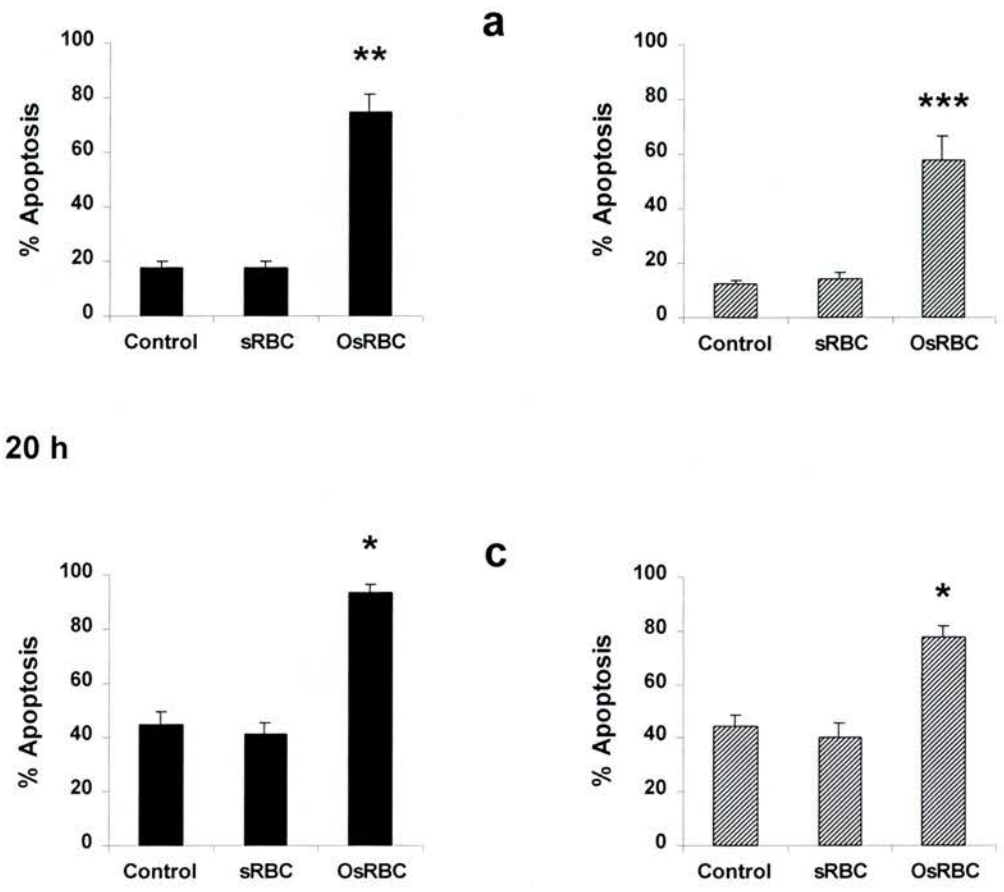


Figure 4.12a,b,c,d: Phagocytosis of OsRBCs stimulates apoptosis in equine neutrophils

Equine neutrophils (5 x 10^6/ml) were incubated in MF at 37°C for 8 (**a** and **b**) or 20 h (**c** and **d**) either in the absence (Control) or presence of non-opsonised (sRBC) or opsonised sheep erythrocytes (OsRBC) prepared as described in Section 2.10.2 and added at a ratio of 3 RBCs per neutrophil. Cells were resuspended and apoptosis (%) assessed morphologically (closed bars) on Diff-Quik stained cytocentrifuge preparations and by Annexin V binding (hatched bars) analysed by flow cytometry.

a: Apoptosis assessed morphologically at 8 h.

b: Apoptosis assessed by Annexin V binding at 8 h.

c: Apoptosis assessed morphologically at 20 h.

d: Apoptosis assessed by Annexin V binding at 20 h.

Values represent mean ± SEM of six separate experiments, each performed in triplicate (morphology) or duplicate (Annexin V binding).

(*; $p<0.01$,**; $p<0.001$,***;$p<0.0001$ compared with time-matched controls)

sites in cells stimulated to undergo apoptosis by phagocytosis remains uncertain. However, careful scrutiny of cytospins identified very few neutrophils with normal morphology, suggesting that Annexin V binding underestimated the true proportion of apoptotic cells.

A time course study of neutrophil apoptosis following phagocytosis of opsonised and control sRBCs was performed and demonstrated an increased rate of morphological apoptosis in OsRBC-stimulated cells at 3 h (Figure 4.13a). Neutrophils appeared only to phagocytose non-opsonised cells after 5 h of co-culture, whereas ingestion of opsonised erythrocytes was maximal by 1 h (Figure 4.13b). This is consistent with other studies in equine neutrophils demonstrating that ingestion of opsonised bacteria is maximal by 30 min (Raidal *et al.*, 1998b).

Obviously, further experiments are required to confirm these observations, but these preliminary findings suggest that phagocytosis-driven apoptosis (or possibly the recognition mechanism involved in ingesting OsRBCs; see Figure 4.13b) could represent a major anti-inflammatory control point in the neutrophil response to microbial challenge. Indeed, study of other opsonins and different prey, such as pathogenic bacteria, would be of great interest in the elucidation of the host response to bacterial disease.

4.2.2.5 Dexamethasone inhibits equine neutrophil apoptosis

Glucocorticosteroids are widely used in the therapy of inflammatory diseases, particularly of the respiratory tract, in both man (McFadden, 1998) and animals. They are widely held as the pharmacological agents of choice in countering the airway inflammation associated with COPD in the horse (Beech, 1991; Robinson, 1997a). Somewhat paradoxically, however, glucocorticosteroids have been shown to inhibit the rate of constitutive human neutrophil apoptosis (Cox, 1995; Liles *et al.*, 1995; Meagher *et al.*, 1996). In this context we evaluated the influence of dexamethasone on apoptosis in equine neutrophils.

Dexamethasone (1 μM) proved to be a potent inhibitor of equine neutrophil apoptosis (Figure 4.14 at both 8 h (% apoptosis; control 15.8 ± 3.0, DEX 3.5 ± 0.8, $p<0.001$, $n = 8$) and 20 h (% apoptosis; control 52.0 ± 4.1, DEX 22.8 ± 1.6, $p<0.0001$, $n = 8$) without affecting cell viability. DNA fragmentation studies

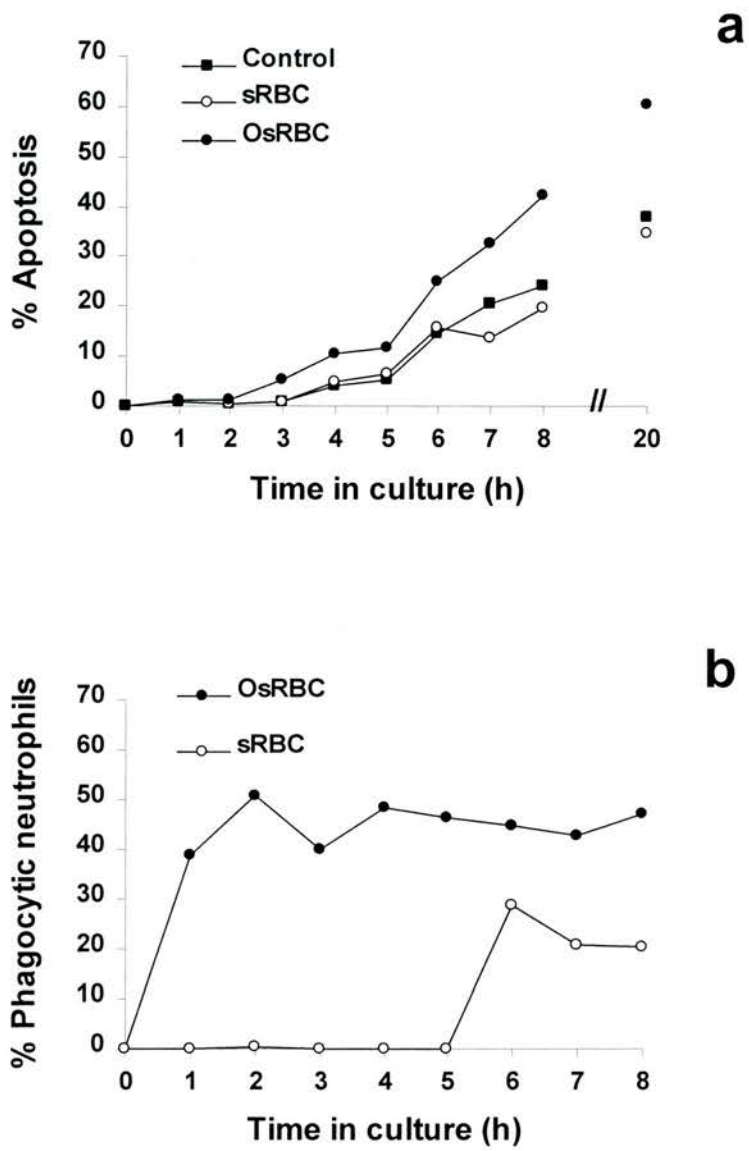


Figure 4.13a,b: Time course of phagocytosis-enhanced apoptosis in equine neutrophils

Equine neutrophils (5 x 10^6/ml) were incubated in MF at 37°C either alone (Control) or in the presence of either non-opsonised (sRBC) or opsonised sheep erythrocytes (OsRBC), prepared as described in Section 2.10.2, at a ratio of 3 cells per neutrophil. Neutrophils were resuspended at the time points indicated.

a: percent apoptosis assessed morphologically on Diff Quik-stained cytocentrifuge preparations.

b: percent of phagocytic neutrophils assessed morphologically on Diff-Quik-stained cytocentrifuge preparations.

Values represent mean of triplicate determinations from a single experiment.

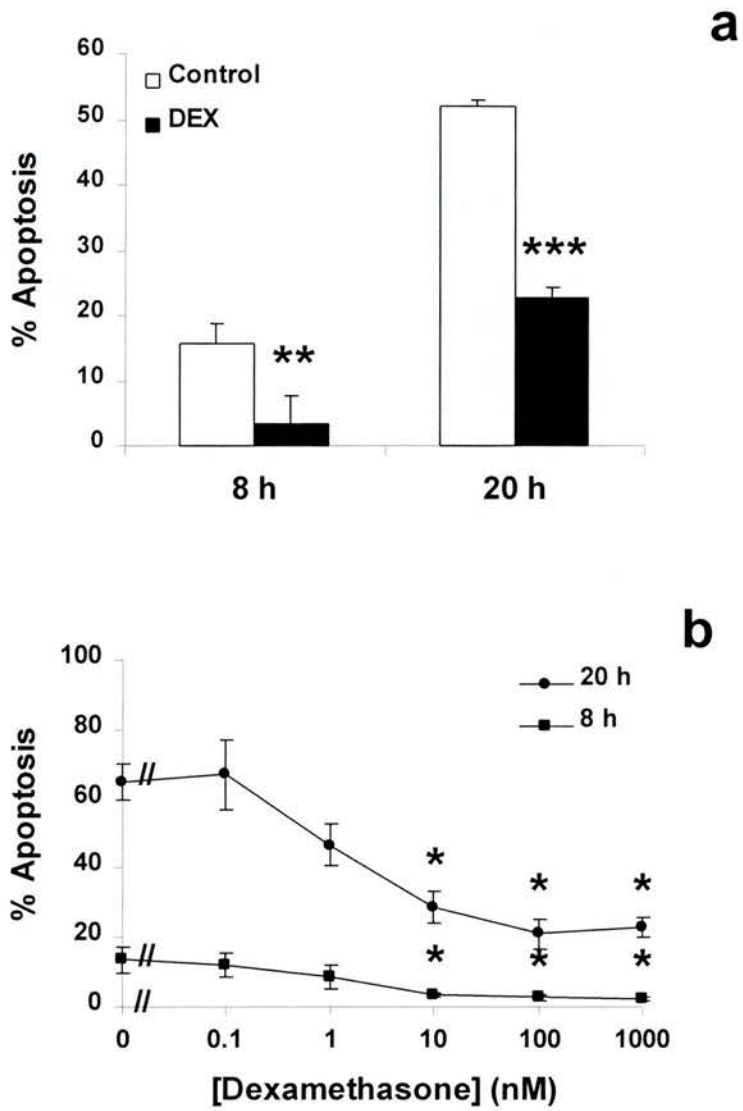


Figure 4.14a,b: Dexamethasone inhibits apoptosis in equine neutrophils

Equine neutrophils (5 x 10^6/ml) were incubated in MF at 37°C for 8 and 20 h. Cells were resuspended and percent apoptosis assessed morphologically on Diff Quik-stained cytocentrifuge preparations.

a: Cells incubated either alone (open bars) or in the presence of 1 μM Dexamethasone (DEX; closed bars). Values represent mean ± SEM of eight separate experiments, each performed in triplicate.

b: Cells incubated, either alone or in the presence of DEX (0.1–1000 nM) for 8 (squares) or 20 h (circles). Values represent mean ± SEM of three separate experiments, each performed in triplicate.

(* ; $p<0.01$,** ; $p<0.001$,*** ;$p<0.0001$ compared with time-matched controls)

provided further qualitative data to confirm the enhanced survival of neutrophils incubated with dexamethasone (data not shown).

Moreover, this effect was concentration-dependent (Figure 4.14b), equine cells being very sensitive to the inhibitory effect of dexamethasone (8h; IC_{50} 0.6 ± 2.6 nM, 20h; IC_{50} 1.1 ± 2.4 nM, n = 3). Although no human cells were examined in parallel, these data suggest that equine neutrophils are significantly more sensitive to the dexamethasone survival signal, since Liles *et al.* (1995) reported an IC_{50} for dexamethasone of 50 nM in human cells when assessed after 24 h in culture.

4.3 DISCUSSION

The light microscopic features of equine neutrophils aged in culture were found to be very similar to those observed in apoptotic human neutrophils (Savill *et al.*, 1989b; Lee *et al.*, 1993). This is not unsurprising given the apparently universal occurrence of the stereotypical features of cytoplasmic condensation, with or without vacuolation, and the marked condensation of nuclear heterochromatin observed in cells undergoing apoptosis or programmed cell death (Kerr *et al.*, 1972; Wyllie *et al.*, 1980b; Kerr *et al.*, 1995; Majno and Joris, 1995;). The time-dependent progression of equine neutrophils manifesting these typical light microscopic and ultrastructural features and the maintenance of plasma membrane integrity confirmed their constitutive ability to undergo apoptosis.

Some of the earliest and most unmistakable morphological features of any cell undergoing apoptosis are found in the nucleus, where condensed chromatin may fill the whole of a nuclear remnant or be confined to distinct peripheral crescents (Kerr *et al.*, 1995; Majno and Joris, 1995). Cytoplasmic vacuoles, although not a consistent morphological feature of apoptosis (Kerr *et al.*, 1995), are recognized in neutrophils (Savill *et al.*, 1989b) and have been associated with the large surface craters observed with scanning electron microscopy in other cell types, suggesting that cells with these features may have expelled part of their contents by exocytosis (Kerr *et al.*, 1995).

Whilst such structural alterations remain the "gold standard" for identification of the apoptotic phenotype, they must not be considered in isolation. In this study parallel confirmatory biochemical evidence was obtained from electrophoresis of DNA. This produced the classical "ladder' pattern of integer multiples of 180–200 base pair nucleosomes (Wyllie, 1980a). This results from activation of an endogenous endonuclease that cleaves DNA in the linker region between histones on the chromosome (Bortner *et al.*, 1995) to produce DNA fragments with 3'-OH and 5'-phosphate ends (Eastman, 1995a). Indeed, 15 years ago Wyllie and co-workers (1984) demonstrated that the condensed chromatin recognized morphologically in apoptotic thymocytes was composed of endogenously fragmented chromatin. The process of DNA cleavage may indeed proceed from large (50 - 300 kbp) fragments followed by degradation into progressively smaller nucleosome multiples (Bortner *et al.*, 1995). This fragmentation tends to be more extensive in haematopoietic cell lineages (Eastman, 1995a), such as neutrophils.

Apoptosis is also associated with a decrease in cell size (Majno and Joris, 1995); hence its early description as "shrinkage necrosis", and although not assessed here, both acridine orange staining (Liles *et al.*, 1995) and flow cytometric analysis (Hachiya *et al.*, 1995) have demonstrated such a reduction in the size of apoptotic human neutrophils. Flow cytometry has also permitted detailed cell-by-cell analysis of changes in both the structure of the plasma membrane and its repertoire of surface molecule expression in neutrophils undergoing apoptosis (Dransfield *et al.*, 1994; Dransfield *et al.*, 1995; Homburg *et al.*, 1995). These changes are highly specific for the apoptotic phenotype. Down-regulation of both the expression and function of certain surface adhesion receptors and ligands is recognized in human neutrophils (Dransfield *et al.*, 1995). For example, there is a tight correlation between reduced expression of the low affinity IgG receptor FcγRIII (CD16) and the appearance of apoptotic morphology. This forms the basis of a sensitive assay for apoptosis in human neutrophils (Dransfield *et al.*, 1994; Homburg *et al.*, 1995). Of particular interest when studying cellular apoptosis in a species such as the horse, for which there is a paucity of well-defined immunological reagents, are the apparently universal changes in plasma membrane phospholipid structure that occur in apoptosis (Martin *et al.*, 1995). Normal blood cell membranes have an asymmetric distribution

of their component phospholipids, with phosphatidylcholine and sphingomyelin predominantly expressed on the outer leaflet and most of the phosphatidylethanolamine and phosphatidylserine being confined to the inner leaflet (Van Meer, 1993). As cells undergo apoptosis, these phospholipids become redistributed (Mower *et al.*, 1994), such that asymmetry is lost and phosphatidylserine becomes exposed on the outer leaflet of the lipid bilayer (Martin *et al.*, 1995). Annexin V, a member of a family of high affinity aminophospholipid binding proteins, preferentially binds phosphatidylserine. The use of FITC-labelled Annexin V has demonstrated consistent early exposure of phosphatidylserine on a wide variety of human and murine cell types undergoing either stimulated or constitutive apoptosis, including neutrophils (Koopman *et al.*, 1994; Homburg *et al.*, 1995; Martin *et al.*, 1995). In most cell types, phospholipid redistribution and the acquisition of Annexin V binding sites precedes chromatin condensation and DNA fragmentation (Mower *et al.*, 1994; Martin *et al.*, 1995). In neutrophils however, these changes appear to be more tightly associated (Homburg *et al.*, 1995). Indeed, as well as having demonstrated that apoptotic equine neutrophils also acquire appropriate binding sites for Annexin V, this event appears to be tightly temporally associated with nuclear condensation.

The importance of assessing apoptosis by a combination of assay methods is highlighted when attempting to define the kinetics of cell death *in vivo*, as not all the classical changes will be apparent prior to the rapid recognition and engulfment of apoptotic cells by phagocytes (Nakamura *et al.*, 1997). Measurement of Annexin V binding may be particularly relevant in this context given the mechanistic involvement of phosphatidylserine exposure in macrophage recognition of the apoptotic phenotype in a number of cells lineages (Fadok *et al.*, 1992; Savill, 1997b). The TUNEL method of *in situ* staining of the exposed 3'-OH ends of fragmented DNA (Ben-Sasson *et al.*, 1995) clearly and specifically identified apoptotic cells in short term (8 h) cultures of equine neutrophils. This was defined *in vitro* with a view to utilising this technique to positively identify fragmented DNA in apparently condensed nuclear material within both free cells and phagocytic macrophages in BALF collected *in vivo*.

When comparing apoptosis data obtained in different studies, it is important to clearly define the cell culture conditions employed because the constitutive rate of neutrophil apoptosis can be profoundly affected by pH (Leblebicioglu and Walters, 1999), cell density, the nature of the culture vessel and the presence or absence of serum or protein in the culture medium (Hannah *et al.*, 1998). Although no direct parallel comparison was made, equine neutrophils appeared to become apoptotic at a somewhat faster initial rate (< 8 h) but thereafter more slowly than human cells (e.g. % apoptosis at 20 h; equine 42.1 ± 1.5, human 52.4 ± 4.8, n = 6). This results in a more linear relationship between the proportion of apoptotic cells and time for equine neutrophils compared with the more sigmoid kinetic relationship for human cells (Savill *et al.*, 1989b). Notwithstanding the part that donor variability might play in determining the constitutive rate of apoptosis (Dransfield *et al.*, 1994), this trend is consistent with the reported circulating half-lives of 10.5 and 6.8 h for equine (Carakostas *et al.*, 1981) and human (Boggs, 1967) neutrophils, respectively. Although more recent studies suggest that these *ex vivo* labelling and re-infusion methods overestimate neutrophil disappearance rates (Bicknell *et al.*, 1994), these early investigations of granulocyte kinetics did provide preliminary evidence that the lifespan of circulating neutrophils is prolonged during an inflammatory response. Lee and colleagues (1993) demonstrated the ability of inflammatory mediators to inhibit apoptosis and prolong the functional longevity of human neutrophils *in vitro*. Many other studies have detailed the regulatory effects of cytokines and other inflammatory mediators on neutrophil longevity (Cohen *et al.*, 1992; Squier *et al.*, 1995; Liles *et al.*, 1996). A unifying hypothesis began to emerge that agents known to prime or activate neutrophil function (Lee *et al.*, 1993; Murray *et al.*, 1997) would retard the constitutive rate of apoptosis. However, this concept was questioned by the lack of such an effect with some priming/activating agents, for example, fMLP and PAF and the demonstration of an early pro-apoptotic effect of TNF-α (Takeda *et al.*, 1993; Murray *et al.*, 1997). Considerable disagreement also exists regarding the effects of certain of these agents on neutrophil apoptosis; hence fMLP for example, has been reported to inhibit (Lee *et al.*, 1993; Watson *et al.*, 1996b), promote (Hebert *et al.*, 1996) or to have no effect on human neutrophil apoptosis (Murray *et al.*, 1997).

This preliminary study in equine neutrophils sought to address the regulation of apoptosis by such inflammatory mediators. The lack of effect of fMLP (1 μM) on equine neutrophil apoptosis is consistent with certain human studies e.g. (Watson *et al.*, 1996b; Murray *et al.*, 1997). However, the former group did identify an inhibitory effect at fMLP concentrations below 100 nM.

Although the direct functional responses of equine neutrophils to LTB_4 are similar to those reported in human cells (see Section 3.1), a promotion of neutrophil survival, as reported in human neutrophils (Hebert *et al.*, 1996), was not observed in equine cells. However, parallel equine and human studies would be necessary to confirm this discrepancy.

Functional responses to hrGM-CSF have been recognized in some equine cell types (Dr D. Horohov, 1998, personal communication) and systemic administration of canine rGM-CSF results in increased myeloid activity and a significant peripheral neutrophilia in foals (Zinkl *et al.*, 1994). However, the consistent concentration-dependent enhancement of survival observed in human neutrophils (Figure 4.7, Cox *et al.*, 1992, Lee *et al.*, 1993} was not observed in equine cells. Failure of equine neutrophil GM-CSF receptors to recognize this particular recombinant cytokine may explain this lack of effect.

The highly variable response of equine cells to TNF-α is difficult to explain, but donor-specific effects may underlie a large part of this variability. Equally variable donor responses are observed in human cells in response to hrTNF-α (Dr. J. Murray, personal communication). Incubation of human neutrophils with LPS abolishes the pro-apoptotic effect of TNF-α (Murray *et al.*, 1997) and exposure of cells in these experiments to even trace amounts of LPS during isolation might mask any TNF-α effect. However, neutrophil priming studies performed in parallel demonstrated that freshly isolated cells were not basally primed (based on their response to fMLP), suggesting that significant LPS contamination did not occur. The highly consistent priming of equine cells by both the human and equine recombinant cytokines (see Sections 3.2.2 and 3.2.3.2) in the absence of a reliable effect on apoptosis, could indicate differential engagement of equine TNF-α receptors by the two recombinant molecules. In human neutrophils, TNF-α-induced priming and the late inhibitory effect on apoptosis are mediated by the TNFR 55, whereas the early pro-apoptotic

effect relies upon ligation of both the TNFR 55 and the TNFR 75 (Murray *et al.*, 1997). Obviously, further detailed experiments will be necessary before the true nature of these responses is apparent. Indeed, identification and cloning of equine TNF-α receptors may be required for further progress to be made on this question. The potent anti-apoptotic effect of ZAS confirmed that inflammatory mediators could indeed activate a respiratory burst and prolong equine neutrophil survival. This agent may prove useful in future studies to dissect the modulation of equine neutrophil apoptosis. However, in future, purification of C5a from ZAS and identification of other pro-inflammatory components of this somewhat crude reagent should be undertaken.

Undoubtedly, the most intriguing and novel data in this study is the marked pro-apoptotic effect of LPS in equine neutrophils. Its concentration-dependence (EC_{50} 32.3 ± 2.4 ng/ml at 20 h) is similar to that determined for priming the respiratory burst (EC_{50} 19.1 ± 4.7 ng/ml). The effect was confirmed on multiple occasions using both morphological and biochemical criteria and subsequent experiments suggested that the response was independent of TNF-α. This effect is diametrically opposed to the profound concentration-dependent inhibitory effect of LPS in human neutrophils (Figure 4.7, (Colotta *et al.*, 1992; Lee *et al.*, 1993; Yamamoto *et al.*, 1993). This was neither an anomaly of the culture system (as LPS enhanced the survival of human cells incubated in parallel, see Figure 4.7) nor a LPS type-specific effect as identical results were also obtained with a rough *S. typhimurium* LPS. Again in contrast to human cells (Keel *et al.*, 1997), this response was not mediated, even in part, by TNF-α. LPS has been reported to play either a direct or facilitatory role in stimulating apoptosis in a number of more long-lived cell types, including lymphocytes (Norimatsu *et al.*, 1995), vascular endothelial cells (Wong *et al.*, 1996) and alveolar macrophages by a ROS-dependent mechanism (Bingisser *et al.*, 1996), but this effect has not been previously recognized in granulocytes. Assessment of apoptosis by multiple methods in response to a larger panel of LPS types and over a more detailed time course will be required to confirm and dissect this novel pro-apoptotic response in equine neutrophils.

In spite of the widespread use of glucocorticosteroids in the treatment of inflammatory disease in the horse, information on the effects of such agents on

neutrophil function *in vitro* is lacking. Just two studies appear to have been published both demonstrating the ability of DEX (1 – 500 μM) to inhibit ZAS-stimulated adhesion of equine neutrophils (Slauson *et al.*, 1987; Bochsler *et al.*, 1990). In contrast, the regulatory effects of glucocorticosteroids on human neutrophil function and apoptosis in particular, are an area of intense study (Cox, 1995; Liles *et al.*, 1995; Meagher *et al.*, 1996; Liu *et al.*, 1999). Equine neutrophils appeared to be even more sensitive than human cells to the dramatic inhibitory effect of DEX on their rates of apoptosis (Liles *et al.*, 1995). Furthermore, quite unlike human neutrophils, which require at least 8 h exposure to DEX before inhibition is seen (Meagher *et al.*, 1996), in equine cells a significant inhibitory effect is already apparent by this time point. In human cells this effect is mimicked by a number of other glucocorticosteroids including cortisol, hydrocortisone, budenoside and methylprednisolone (Cox, 1995) by a receptor-mediated mechanism (Meagher *et al.*, 1996). However, unlike many other anti-apoptotic stimuli, such as GM-CSF, DEX does not prime neutrophils for an enhanced respiratory burst (Cox, 1995). An inter-species difference in the response of equine neutrophils to DEX was also recognized by Slauson *et al.* (1987), wherein DEX inhibited C5a-induced adhesion; in human neutrophils it does not. The mechanism of DEX inhibition of neutrophil apoptosis is currently unknown, but as human cells require at least 8 h of exposure to DEX before an inhibitory effect is revealed, drug-induced synthesis of an anti-apoptotic protein seems unlikely (Meagher *et al.*, 1996). Whether a different or complimentary mechanism underlies the response of equine neutrophils is a subject for future study. The dramatic increase in peripheral neutrophil counts observed in both humans and horses (Lassen and Swardson, 1995) following systemic administration of glucocorticosteroids may be mediated, in part, by directly delaying neutrophil apoptosis and clearance (Meagher *et al.*, 1996). More importantly, this enhanced longevity of DEX-exposed neutrophils may have major implications regarding the clearance of these cells from an inflammatory site and could well be detrimental in terms of the resolution of inflammation *in vivo*. However, this consideration is balanced by the work of Liu and colleagues (1999), who observed a major potentiation in the non-phlogistic phagocytosis of apoptotic neutrophils by glucocorticoid-treated macrophages. This suggests that corticosteroid therapy may

also positively promote the resolution of inflammation. If macrophage uptake of apoptotic neutrophils is rate limiting, this effect may play an important part in the reduction in BALF neutrophil count observed in horses with RAO following administration of either aerosol beclomethasone dipropionate or parenteral dexamethasone (Rush *et al.*, 1998a).

Phagocytosis of opsonised particles is a key neutrophil effector function in host defence and this produced a striking promotion of apoptosis in equine neutrophils. A similar response has been observed in human neutrophils following ingestion of both complement (Coxon *et al.*, 1996; Watson *et al.*, 1996a; Watson *et al.*, 1996b) and IgG opsonised targets (Gamberale *et al.*, 1998). These effects were found to be dependent upon ROS generation during a phagocytosis-induced respiratory burst. Investigation of this response in equine cells identified no pro-apoptotic effect after the delayed ingestion of non-opsonised sRBC, suggesting that the mechanism of particle recognition and uptake is crucial in determining the cell's response. By selectively blocking Fc receptors and phagocytosis with cytochalasin B, Gamberale and colleagues (1998) demonstrated that particle ingestion was superfluous and that apoptosis was also stimulated in association with a prolonged respiratory burst following binding to FcγIIa receptors. In a single experiment performed with equine neutrophils, cytochalasin B at a concentration of 5 μg/ml (shown not to affect apoptosis in the human study) enhanced apoptosis in equine cells, preventing meaningful interpretation of the importance of ingestion *per se* in phagocytosis-stimulated apoptosis (data not shown).

Whilst many of the foregoing studies reporting the modulation of constitutive neutrophil apoptosis give clues to a number of key players, the intracellular mechanisms underlying apoptosis and its regulation remain elusive. Undoubtedly, constant protein synthesis is necessary to maintain the neutrophil's brief lifespan, as evidenced by the rapid induction of apoptosis following exposure to the protein synthesis inhibitor, cycloheximide (Tsuchida *et al.*, 1995; Whyte *et al.*, 1997). Alterations in intracellular ion balance leading to cell acidification in association with apoptosis (Cohen *et al.*, 1992; Eastman, 1995b) have not been recognized in the neutrophil. Indeed, transient elevations in cytosolic free calcium ions, induced by low concentrations of the calcium ionophore A23187, retarded apoptosis and

modulation of intracellular pH had no effect on neutrophil apoptosis (Whyte *et al.*, 1993a). However, a subsequent study suggested a causal relationship between the appearance of apoptotic morphology in human neutrophils and an early and rapid fall in pH (Gottlieb *et al.*, 1995). Moreover, these authors suggested that G-CSF inhibited apoptosis by upregulation of an H^+-ATPase proton export pump thereby preventing cell acidification.

Liles and co-workers (Liles *et al.*, 1996) have proposed an autocrine mechanism related to co-expression of the TNF/nerve growth factor family member Fas (CD 95) and its ligand (FasL) on the neutrophil's surface to explain the cell's inexorable progression toward death. Fas/FasL ligation is well recognized as a trigger for the clonal deletion of autoreactive T cells, and both activation-induced T cell suicide and cytotoxic T cell killing (Nagata and Golstein, 1995). Also Fas-mediated neutrophil death is inhibited by well-described anti-apoptotic stimuli such as pro-inflammatory cytokines, dexamethasone and tyrosine kinase inhibitors (Liles *et al.*, 1996). Human neutrophil apoptosis is abrogated by tyrosine phosphatase inhibitors, indicating a central role for tyrosine phosphorylation in apoptosis signalling (Yousefi *et al.*, 1994). Recently, constitutive tyrosine phosphorylation and activation of the serine/threonine kinase, p38-mitogen-activated protein kinase (p38-MAPK), a protein kinase generally associated with cell survival signals (Eastman, 1995b), has been identified in freshly isolated neutrophils and implicated in constitutive apoptosis (Aoshiba *et al.*, 1999). These workers also implicated constitutive intracellular ROS generation, downstream to p38-MAPK, in the spontaneous death programme. This supports other recent work demonstrating that changes in intracellular redox status may be critical in the regulation of both constitutive and stimulated neutrophil apoptosis (Narayanan *et al.*, 1997; Oishi and Machida, 1997; Watson *et al.*, 1997a).

As alluded to earlier, the onset of apoptosis is associated with down-regulation of cellular functions. Equine neutrophils showed a dramatically reduced respiratory burst in response to a receptor-mediated stimulus (ZAP); the magnitude of this reduction (80%) being substantially greater than the proportion of morphologically apoptotic cells (50%). This could suggest that the cells become functionally isolated from their surroundings at an early stage in the apoptotic programme, prior to the

development of typical morphologic features of apoptosis. These data concur with findings in human neutrophils, where reductions in both basal (spreading on glass and shape change) and stimulated responses (shape change, chemotaxis, respiratory burst and degranulation) to receptor-mediated agonists (fMLP, C5a and opsonised zymosan) closely mirrored levels of apoptosis in elutriation fractions of aged cells (Whyte *et al.*, 1993b). However, some of the data from the study by Whyte and colleagues (1993) also indicated that the fall off in functional responses was greater than the proportion of visibly apoptotic cells. These authors went on to demonstrate by autoradiography that this was associated with a loss of functional fMLP receptors, with fewer than 1% of apoptotic cells binding significant amounts of [^{3}H] fMLP. This may indicate some degree of receptor cross talk in the global down-regulation of receptor-mediated function, given the association between reduced CD16 expression and apoptosis (Dransfield *et al.*, 1994) and the functional linkage of this receptor with that for fMLP (Kew *et al.*, 1992).

However, in contrast to the effect of a receptor-mediated stimulus, direct activation of PKC and superoxide anion generation in response to PMA was maintained in aged equine neutrophils. This suggests that the signal transduction mechanisms regulating NADPH assembly are intact in apoptotic cells, but are inaccessible to receptor-mediated stimuli. PMA-stimulated superoxide anion release is also maintained in apoptotic human neutrophils (Whyte *et al.*, 1993b; Narayanan *et al.*, 1997). However, recent, more detailed studies suggest that the regulation of intracellular oxidative activity in apoptotic neutrophils is much more complex than these initial experiments might indicate (Narayanan *et al.*, 1997). Narayanan and co-workers (1997) demonstrated a time-dependent reduction in PMA-stimulated H_2O_2 production in contrast to the maintenance of superoxide anion generation, suggesting that the stability of the PMA response was pathway specific. Moreover, they observed a fall in intracellular glutathione and superoxide dismutase content in cells undergoing apoptosis, again implicating a disruption of redox status in both the apoptotic programme and the associated down-regulation of oxidative capacity.

The changes in receptor number and function that accompany apoptosis, have many implications for the limitation of neutrophil-mediated tissue injury *in vivo*. Notably, apoptotic neutrophils show a reduction in both selectin- and integrin-mediated

adhesion events associated with both decreased adhesion molecule/ligand expression (selectins) and function (integrins) (Dransfield *et al.*, 1995). As adhesion *per se* can augment pro-inflammatory neutrophil functions (Schleiffenbaum and Fehr, 1990; Anderson, 1995), the altered adhesive capacity of apoptotic cells may also serve to limit release of their histotoxic contents.

Commitment to apoptosis as a means of limiting the pro-inflammatory potential of neutrophils at an inflamed site presumes that resident phagocytes will clear these cells. However, if this clearance route is inadequate or inoperable, impaired functional responses to physiological stimuli may serve as a short-term brake on the neutrophil's latent capacity to cause collateral cellular injury. Although prolongation of neutrophil longevity by inflammatory mediators runs the risk of enhanced neutrophil-mediated damage, this may also serve to maintain antimicrobial defences and neutrophil integrity whilst awaiting recruitment of appropriate clearance routes prior to commitment to apoptosis.

This study has served to initiate understanding of apoptosis and its regulation in equine neutrophils and has established the potential of this programme of cell death to play a central role in a resolution of inflammation favouring minimal host tissue damage. Furthermore, the marked difference in the responses to LPS and the difference in sensitivity and time dependence to DEX in equine cells in comparison to those established for human neutrophils may be of value in dissecting the underlying regulatory mechanisms of apoptosis in neutrophils.

CHAPTER 5

CLINICAL, BRONCHOSCOPIC AND PERIPHERAL BLOOD RESPONSES OF COPD - SUSCEPTIBLE HORSES TO HAY / STRAW CHALLENGE

5.1 INTRODUCTION

Although the precise aetiology of equine COPD remains elusive (Section 1.1.3) the association between the development of lung dysfunction and the use of poorly saved or frankly mouldy straw and hay is well recognized. Critical evaluation of horses with evidence of chronic pulmonary disease has demonstrated the difficulty in making a definitive diagnosis of COPD based on clinical signs alone (McPherson *et al.*, 1978; Mair, 1987a; Naylor *et al.*, 1992; Dixon *et al.*, 1995a). Attempts to definitively diagnose COPD based on strict physiological criteria documenting impaired pulmonary mechanics and gas exchange (Sasse, 1971; McPherson *et al.*, 1978) have been shown to be too insensitive to detect less severely affected animals (Mair, 1987a; Dixon *et al.*, 1995c).

Early pathological studies identified the pulmonary inflammation in COPD as a chronic neutrophilic bronchiolitis (Section 1.1.4) and the introduction of routine respiratory cytology to equine medicine has revolutionised the diagnosis of chronic pulmonary diseases (Whitwell and Greet, 1984; Mair, 1987a; Mair *et al.*, 1987b). Cytological evaluation of tracheal secretions (Nuytten *et al.*, 1983; Larson and Busch, 1985; Mair, 1987a) and more importantly BALF confirmed that the pulmonary inflammation in affected animals was invariably associated with recruitment of neutrophils to the airspaces (Derksen *et al.*, 1985b; Naylor *et al.*, 1992; McGorum *et al.*, 1993d).

More detailed investigations utilising short term (5-7 h) experimental hay/straw challenges showed deterioration in pulmonary mechanics within 90 min, arterial hypoxaemia and recruitment of neutrophils (but not eosinophils or platelets) to the

lung within 3-5 h (Fairbairn *et al.*, 1991; Fairbairn *et al.*, 1993; McGorum *et al.*, 1993d).

Despite the consistent presence of neutrophilic pulmonary inflammation in equine COPD (Robinson *et al.*, 1996) and the potential for neutrophils to cause lung injury (reviewed in Section 1.6), there is a relative paucity of information on the functional status of these cells either within the airspaces or in the circulating granulocyte pool of affected animals. Although some studies have begun to address the function of blood and airspace neutrophils in COPD-affected horses (Nuytten *et al.*, 1983; Olszewski and Laber, 1993; Klucinski *et al.*, 1994; Marr *et al.*, 1997a; Olszewski *et al.*, 1999), the true pathogenetic role of the neutrophil in COPD remains unknown (Derksen, 1993; Robinson *et al.*, 1996).

Since its initial description as an *in vitro* phenomenon (Guthrie *et al.*, 1984), neutrophil priming has been shown to be a necessary step in the sequestration of neutrophils in the lung (Worthen *et al.*, 1987; Ussov *et al.*, 1996) and to play a critical role in endothelial cell injury (Smedly *et al.*, 1986; Worthen *et al.*, 1987). However, systemic priming alone, although causing retention of neutrophils in the pulmonary vascular or "marginated" pool, does not necessarily lead to migration into the lung interstitium or lung damage (Ussov *et al.*, 1996).

Having characterised priming and activation of equine neutrophils *in vitro* (see Chapter 3), the same CL assay was used to assess the functional status of blood and airspace neutrophils in the COPD lung inflammation model. Hence, because only primed equine neutrophils undergo respiratory burst activity when challenged with fMLP, it was hoped that the *ex vivo* fMLP-stimulated CL response of neutrophils harvested during hay/straw challenge could be employed as an index of *in vivo* "functional" priming. Indeed, this is the first study to specifically address the question of *in vivo* neutrophil priming in the horse.

Experimental findings regarding the kinetics and functional status of neutrophils harvested from the airspaces following hay/straw challenge and a discussion of this data are presented in Chapter 6.

To date, it has not been possible to establish a direct causative association between the lung injury observed in equine COPD (Kaup *et al.*, 1990b; Votion *et al.*, 1999; Votion *et al.*, 1999) and the presence of neutrophils in the lung using either nuclear

medicine studies (Fairbairn *et al.*, 1993) or experiments relying on recovery of neutrophils from within the airspaces by BAL (McGorum *et al.*, 1993d). Several studies in human patients have suggested a causal relationship between the functional status of circulating neutrophils and lung disease by establishing a significant statistical correlation between the agonist-stimulated respiratory burst activity of circulating neutrophils and both airway hyperresponsiveness in asthma (Meltzer *et al.*, 1989) and lung injury in ARDS (Zimmerman *et al.*, 1983; Zimmerman *et al.*, 1984; Chollet-Martin *et al.*, 1992). However, these conclusions seem less robust in light of studies demonstrating firstly, that neutrophils can undoubtedly be primed and migrate into the airways and not undergo a respiratory burst (Jones *et al.*, 1997) or cause lung injury (Martin *et al.*, 1989; Garat *et al.*, 1995) and secondly, that in patients with extrapulmonary inflammatory diseases, priming is predictive for sequestration in the pulmonary vasculature but does not necessarily lead to transmigration and lung injury (Ussov *et al.*, 1996). It is also possible that changes in blood neutrophil function may be a consequence of lung inflammation in the same way that extrapulmonary diseases such as massive trauma (Botha *et al.*, 1995) and inflammatory bowel disease and vasculitis (Ussov *et al.*, 1996) cause systemic neutrophil priming. Notwithstanding these controversies, investigation of peripheral blood neutrophil kinetics and function in an *in vivo* experimental model was an initial step toward evaluating the role of neutrophils in equine COPD and assessing whether systemic neutrophil priming/activation occurred in this disease.

5.2 RESULTS

5.2.1 CHARACTERISATION OF COPD – SUSCEPTIBLE HORSES

All six horses recruited for the *in vivo* challenge studies had a prolonged history (> 6 months) of recurrent episodes of airway disease when exposed to poorly saved hay and/or straw. The clinical signs of airway disease reported by the animals' owners included coughing, bilateral mucopurulent nasal discharge, dyspnoea and exercise intolerance. These signs resolved when the animals were kept at pasture or

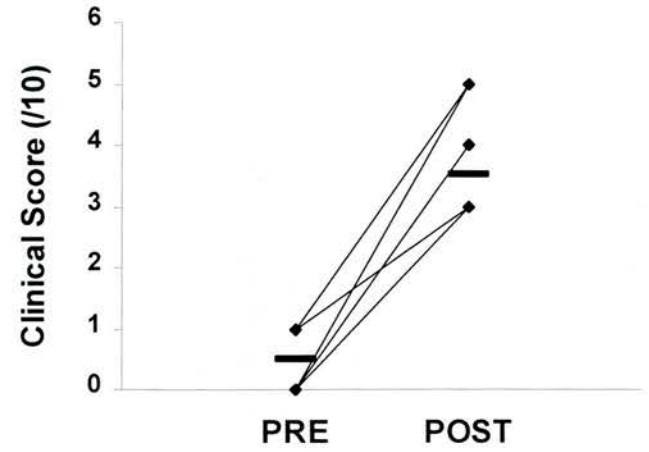
maintained in a "controlled environment". All animals were confirmed to be in remission as assessed by combined clinical examination (clinical score not greater than 1) and examination of BALF cytology (% neutrophils < 5%). To confirm their susceptibility to COPD, all animals underwent a test hay/straw challenge as described in Section 2.7.2. After challenge, all animals had an increased clinical score and an elevated proportion of neutrophils in their BALF (Figure 5.1a,b), thus confirming their disease status.

5.2.2 EFFECTS OF SHAM SERIAL BRONCHOALVEOLAR LAVAGE

The series of sham BAL procedures (see Section 2.7.3) performed whilst the animals were maintained in a controlled environment, had no effect on clinical scores at subsequent BAL collection times ($p>0.05$, $n = 6$, data not shown). Serial BAL alone, also had no significant effect on either peripheral blood neutrophil numbers ($p>0.05$, $n = 3$, data not shown) or PBS-, fMLP-, ZAP- and PMA-stimulated Luci- and Lum-DCL responses ($p>0.05$, $n = 3$, data not shown).

5.2.3 EFFECT OF CHALLENGE ON CLINICAL SCORES

Hay/straw challenge induced a significant increase in clinical scores compared to baseline values at 5 h, 24 h and 4 days after challenge ($p<0.05$, $n = 6$, Figure 5.2). Clinical scores immediately prior to challenge (0 h) were not significantly different from baseline. Clinical scores were greatest at 24 h, but were not statistically different from either the 5 h or the 4 day time points. Scrutiny of the individual components of the clinical score indicated that abnormal respiratory sounds detected over both the distal cervical trachea (6/6 horses at 5 and 24 h after challenge) and the lung fields (6/6 horses at 5 and 24 h) and dyspnoea, characterized by an increased abdominal expiratory effort (5/6 horses at 5 and 24 h), were the most consistent clinical features of the induced respiratory disease.



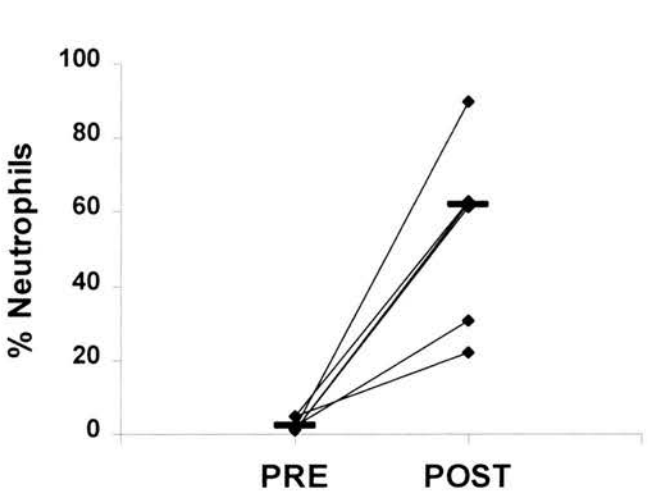


Figure 5.1a,b: Confirmation of COPD-susceptible status of experimental horses
Following an 8 week period at pasture or in a controlled environment horses were challenged as described in Section 2.7.2 for 5 h (3 animals) or 8 h (3 animals) followed by clinical examination and collection and cytological evaluation of BALF.
a: Clinical scores computed immediately before (PRE) and after (POST) a 5 or 8 h challenge as described in Section 2.7.2 and Table 2.3. Horizontal bars represent median values.
b: Proportion of neutrophils (%) in BALF collected 10 days before (PRE) and immediately after (POST) a 5 or 8 h challenge as described in Section 2.7.2. Horizontal bars represent median values.

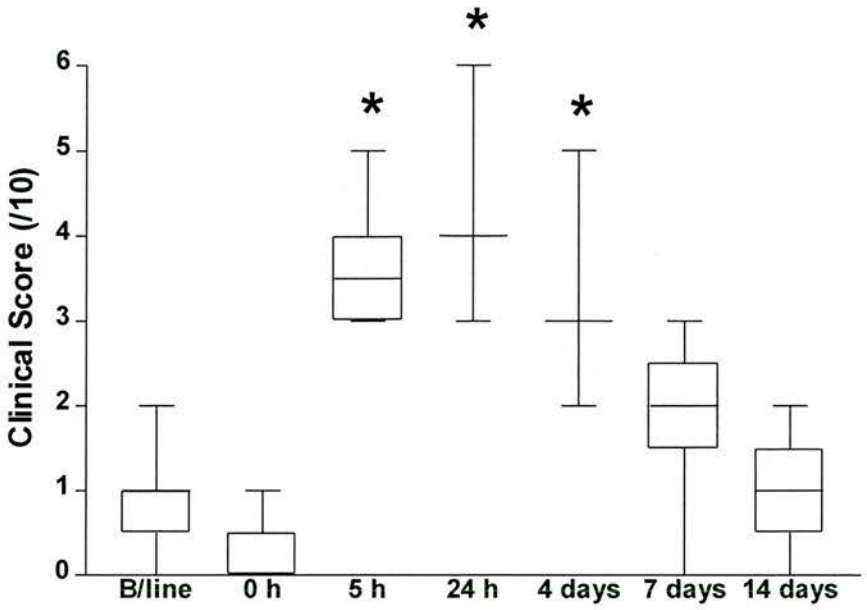


Figure 5.2: Effect of hay/straw challenge on clinical score in COPD-susceptible horses
Clinical scores were computed as described in Section 2.7.2 and Table 2.3 at the time points indicated. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. B/line; baseline examination 10 days prior to challenge; h (hours) and days after the initiation of challenge. (*; $p<0.05$ compared to baseline values, n = 6)

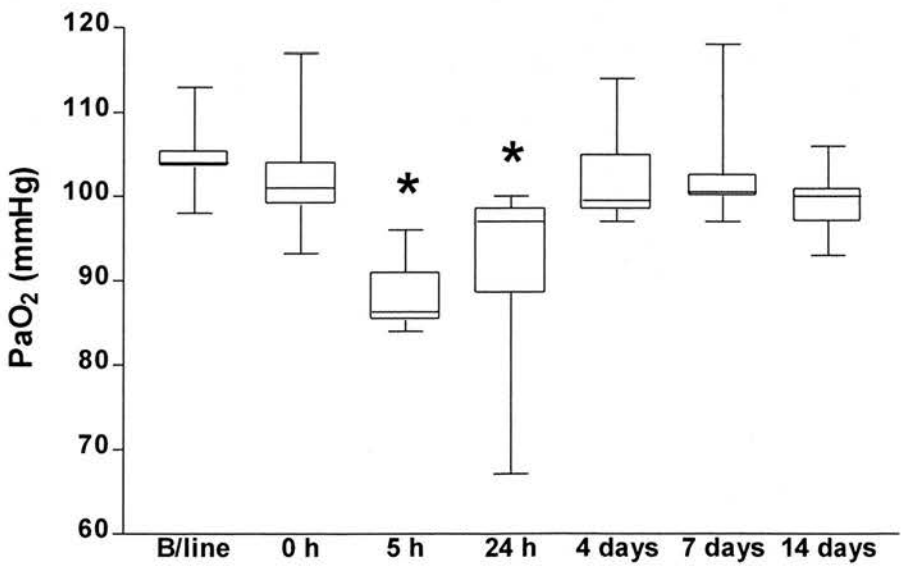


Figure 5.3: Effect of hay/straw challenge on PaO_2 in COPD-susceptible horses
Arterial blood was collected, transported and analysed as described in Section 2.7.2 at the time points indicated. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (*; $p<0.05$ compared to baseline values, n = 6)

5.2.4 EFFECT OF CHALLENGE ON ARTERIAL BLOOD GASES AND pH

Arterial blood gas and pH analyses demonstrated significant hypoxaemia at 5 and 24 h after challenge compared to baseline values (baseline, 104 mmHg, 98 – 113 mmHg (median and range); 5 h, 86.3 mmHg, 84 – 95 mmHg; 24 h, 97 mmHg, 67.1 – 100 mmHg, $p<0.05$, $n = 6$, Figure 5.3). PaO_2 values immediately prior to challenge (0 h) were not significantly different from baseline. Arterial blood pH and $PaCO_2$ were unaffected by challenge.

5.2.5 EFFECT OF CHALLENGE ON THE VOLUME OF TRACHEAL SECRETIONS

The volume and nature of tracheal secretions were observed endoscopically and scored subjectively at the prescribed time points as described in Section 2.7.4. At baseline, four animals had grade 1 secretions (a few flecks) that were tenacious and mucopurulent in nature, the remaining two had no visible secretions. After challenge, all animals had copious tracheal secretions that were of a non-viscous, mucopurulent nature. The volume of secretions was significantly increased in comparison to baseline at 5 h and 24 h after challenge ($p<0.05$, $n = 6$, Figure 5.4) and then gradually reduced over the 14 days after challenge.

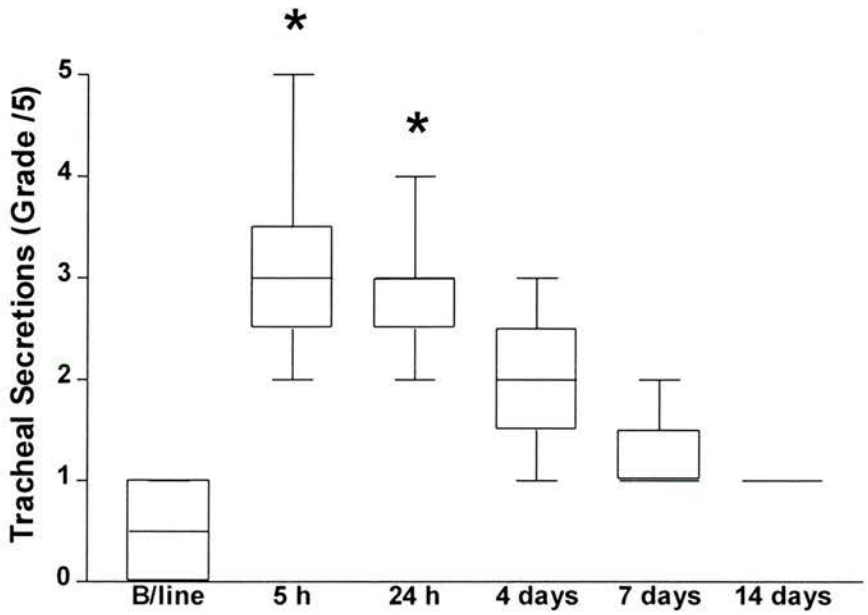


Figure 5.4: Effect of hay/straw challenge on the volume of tracheal secretions in COPD-susceptible horses

The volume of tracheal secretions was graded semi-quantitatively (grade 0-5) as described in section 2.7.4 at the time points indicated. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

5.2.6 EFFECT OF HAY/STRAW CHALLENGE ON PERIPHERAL BLOOD LEUCOCYTE COUNTS

Total and differential leucocyte counts were performed on jugular venous blood samples as described in Section 2.1. All haematological parameters measured immediately prior to challenge (0 h) were not significantly different from baseline. A significant increase in total leucocyte count was present 24 h after challenge ($p<0.05$, $n = 6$, Figure 5.5a) that was due solely to a significant increase in absolute neutrophil counts ($p<0.05$, $n = 6$, Figure 5.5b). This was mirrored by an increase in differential neutrophil counts. Absolute and differential lymphocyte, monocyte, eosinophil and basophil counts were unchanged following challenge (data not shown).

5.2.7 EFFECT OF CHALLENGE ON THE RATE OF CONSTITUTIVE APOPTOSIS IN PERIPHERAL BLOOD NEUTROPHILS

Neutrophils were isolated from peripheral blood and cultured as described in Section 2.6.1. Neutrophil apoptosis was quantified (Section 2.6.2) in cells cultured for 8 and 20 h. The constitutive rate of apoptosis was inhibited (in comparison to baseline values) after 20 h in culture in cells collected 24 h after challenge (% apoptosis at 20 h: baseline, 58.8, 41.4 - 67.5 (median and range); 24 h post-challenge, 42.6, 31.3 – 56.9, $p<0.05$, $n = 6$, Figure 5.6).

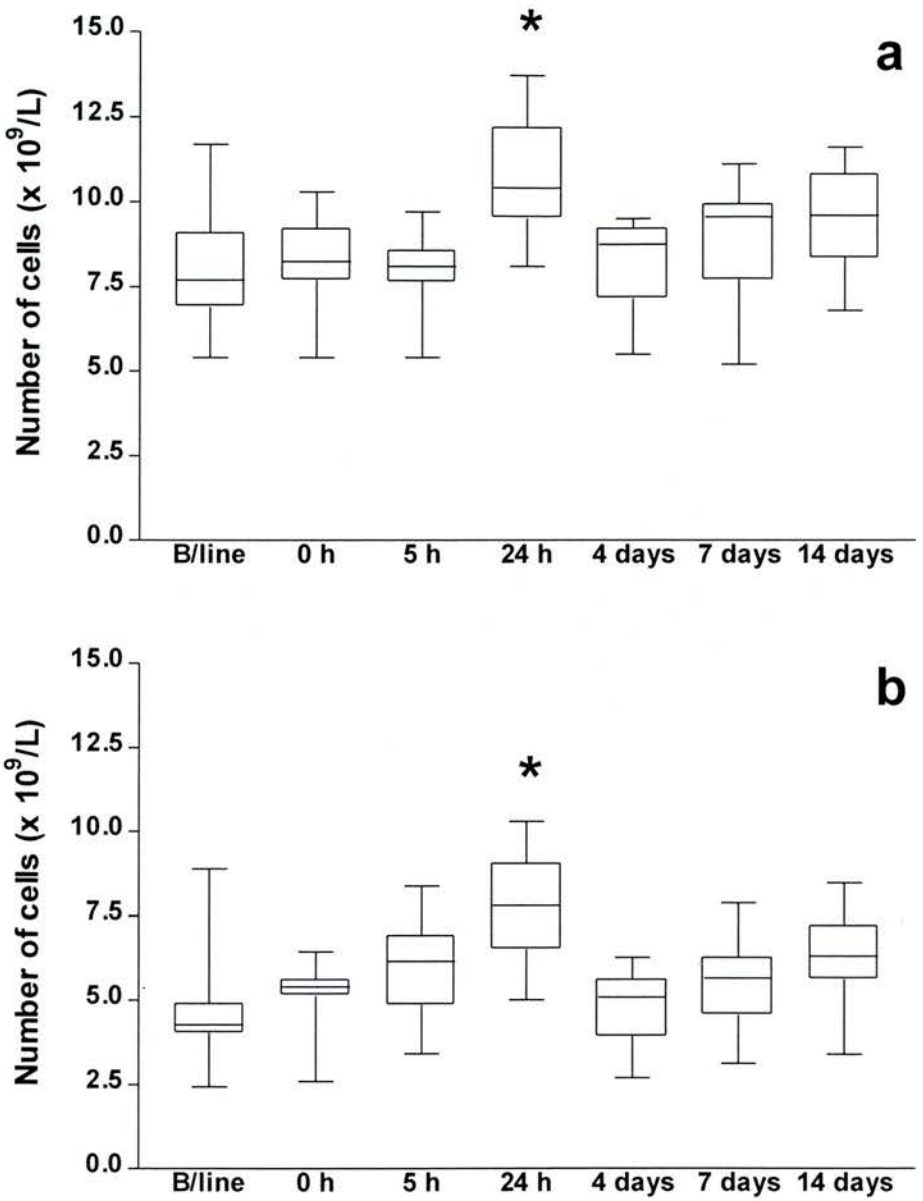


Figure 5.5a,b: Effect of hay/straw challenge on total leucocyte and absolute neutrophil counts in the peripheral blood of COPD-susceptible horses

Total and differential leucocyte counts were performed on jugular venous blood samples as described in Section 2.1 at the time points indicated. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

a: Total leucocyte counts

b: Absolute neutrophil counts

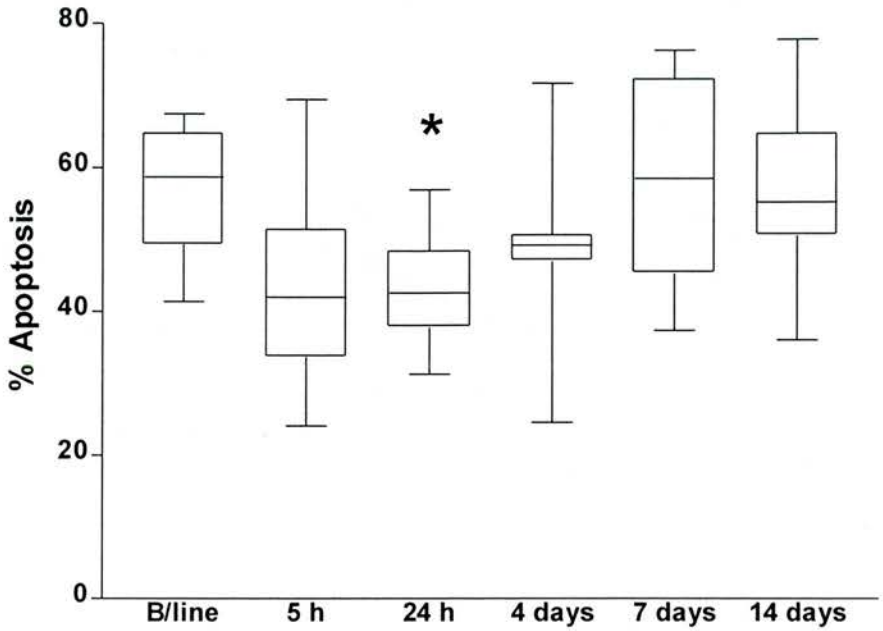


Figure 5.6: Effect of hay/straw challenge on the rate of constitutive apoptosis in peripheral blood neutrophils from COPD-susceptible horses

Neutrophils were isolated at the time points indicated and incubated in MF (5 x 10^6/ml) at 37°C for 20 h. Cells were resuspended and percent apoptosis assessed morphologically on Diff-Quik stained cytocentrifuge preparations in triplicate. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

5.2.8 EFFECT OF CHALLENGE ON PERIPHERAL BLOOD NEUTROPHIL CHEMILUMINESCENCE

Chemiluminescence of neutrophils isolated 10 days before (baseline) and 5 h, 24 h and 4, 7, and 14 days after the 5 h hay/straw challenge was measured in the presence of lucigenin (Luci-DCL) and luminol (Lum-DCL) in response to PBS (Basal), fMLP (1μM), ZAP (10% v/v) or PMA (100 ng/ml) as described in Section 2.8.4.

5.2.8.1 Basal chemiluminescence

Basal Luci- and Lum-DCL of neutrophils isolated 24 h after challenge was significantly greater than baseline when CL was measured in parallel with fMLP over a 5 min time course ($p<0.05$, $n = 6$, basal Luci-DCL; Figure 5.7a). However, basal (unstimulated) data recorded in parallel with ZAP and PMA over a 90 min time course showed that, although there was a trend toward an increased response at 24 h, there was no significant difference in either basal Luci- or Lum-DCL of neutrophils in comparison to baseline ($p>0.05$, $n = 6$) at any post-challenge time point. There was however, considerable inter-animal variation in the basal Luci- (Figure 5.7a) and Lum-DCL (data not shown) responses measured over the 5 min time course at all time points (see box and whisker plots for all time points in Figure 5.7a). This variability must cast doubt on the biological relevance of the statistical inference of a significant increase in basal Luci-DCL at 24 h.

5.2.8.2 fMLP-stimulated chemiluminescence

At 24 h after challenge, fMLP-stimulated Luci-DCL was significantly greater than the baseline fMLP-stimulated response ($p<0.05$, $n = 6$, Figure 5.7b) and significantly greater than basal (unstimulated) Luci-DCL ($p<0.05$) at the same time point. This indicated that circulating neutrophils were primed *in vivo* following hay/straw challenge. There was a trend toward enhanced fMLP-stimulated Luci-DCL 4 days after challenge but this was not significant. At 4 days after challenge however, fMLP-stimulated Lum-DCL was significantly increased in comparison to both the baseline response and unstimulated (PBS) Lum-DCL at that time point ($p<0.05$, $n = 6$, data not shown). FMLP-stimulated Lum-DCL was increased in 4/6 horses at 24 h

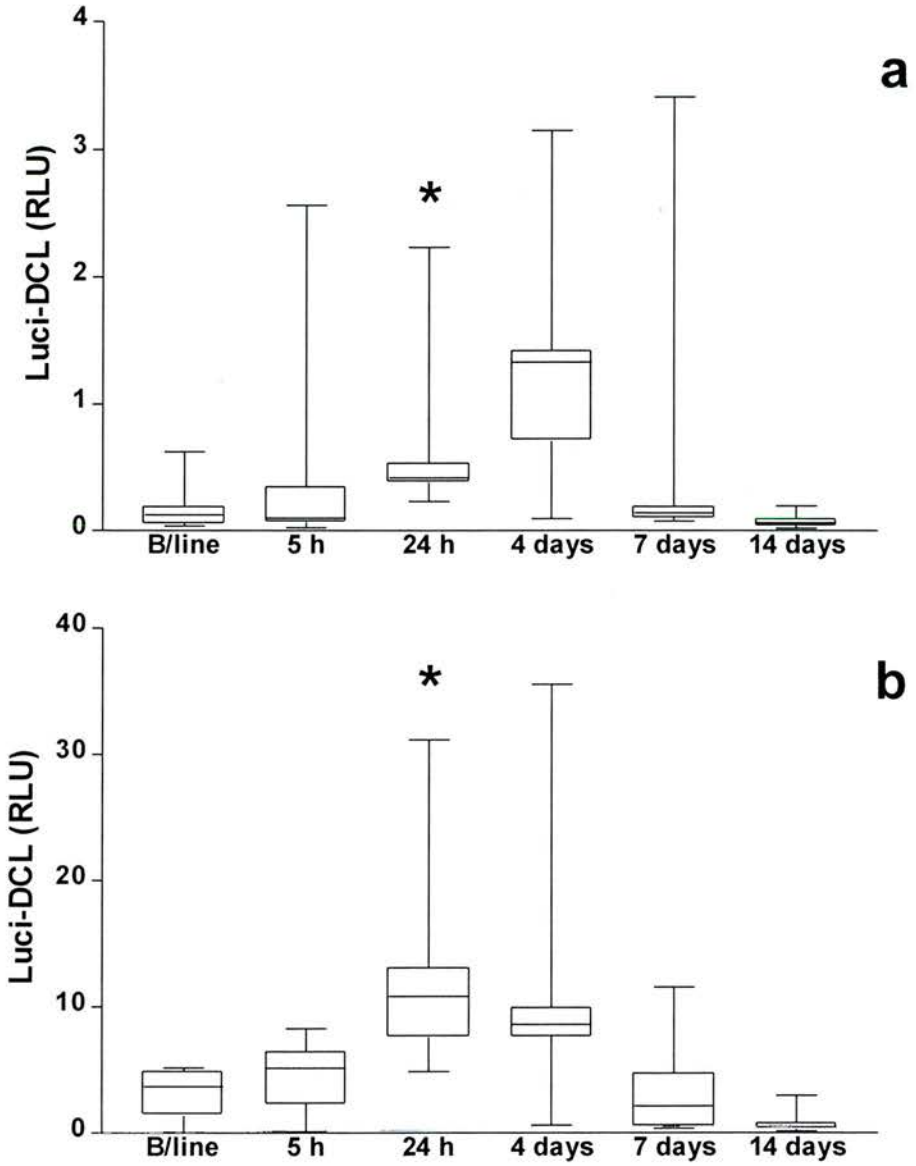


Figure 5.7a,b: Effect of hay/straw challenge on basal and fMLP-stimulated Luci-DCL in peripheral blood neutrophils from COPD-susceptible animals

Basal (unstimulated) and fMLP-stimulated (1 μM) Luci-DCL of peripheral blood neutrophils isolated 10 days before and at the time points indicated after challenge were measured over a 5 min time course. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

a: Basal Luci-DCL (unstimulated); note scale (0-4 RLU).

b: fMLP-stimulated Luci-DCL; note scale (0-40 RLU).

but as a group this response was not significantly greater than at baseline.
In conclusion, these data suggest that peripheral blood neutrophils are primed at 24 h following hay/straw challenge, thus facilitating functional coupling of fMLP receptors.

5.2.8.3 PMA- and ZAP-stimulated chemiluminescence

Both Luci- and Lum-DCL in response to PMA were significantly greater than baseline values 24 h after challenge ($p<0.05$, $n = 6$, Figure 5.8a). Interestingly PMA-stimulated Lum-DCL was significantly lower than baseline at 5 h after challenge ($p<0.05$, $n = 6$, Figure 5.8b). ZAP-stimulated Luci- and Lum-DCL of peripheral blood neutrophils was not different to that measured at baseline at any time point after challenge (data not shown).

5.3 DISCUSSION

Experimental animals were defined as being affected with COPD based on a prolonged clinical history of pulmonary disease and manifestation of clinical signs of airway obstruction and the presence of more than 5% neutrophils in their BALF following hay/straw challenge, as previously described by other workers in this laboratory (McGorum *et al.*, 1993b; Dixon *et al.*, 1995a). Although the precise case definition of COPD has varied between laboratories, the criteria employed herein are very similar to those recently agreed by consensus at an international workshop, at which all major groups involved in equine pulmonary research were represented. This consensus defined equine COPD as a chronic (>3 months duration) pulmonary disease, characterized by recurrent episodes of lower airway obstruction, that was in part, immediately reversible on administration of bronchodilators and accompanied by greater than 5% neutrophils in BALF (Dixon, 1998).
COPD is a disease of middle-aged to older horses with reported median ages of affected animals ranging from 9-13 years (Naylor *et al.*, 1992; Dixon *et al.*, 1995b; Ainsworth, 1999). The horses studied herein had a median age of 17 years. This

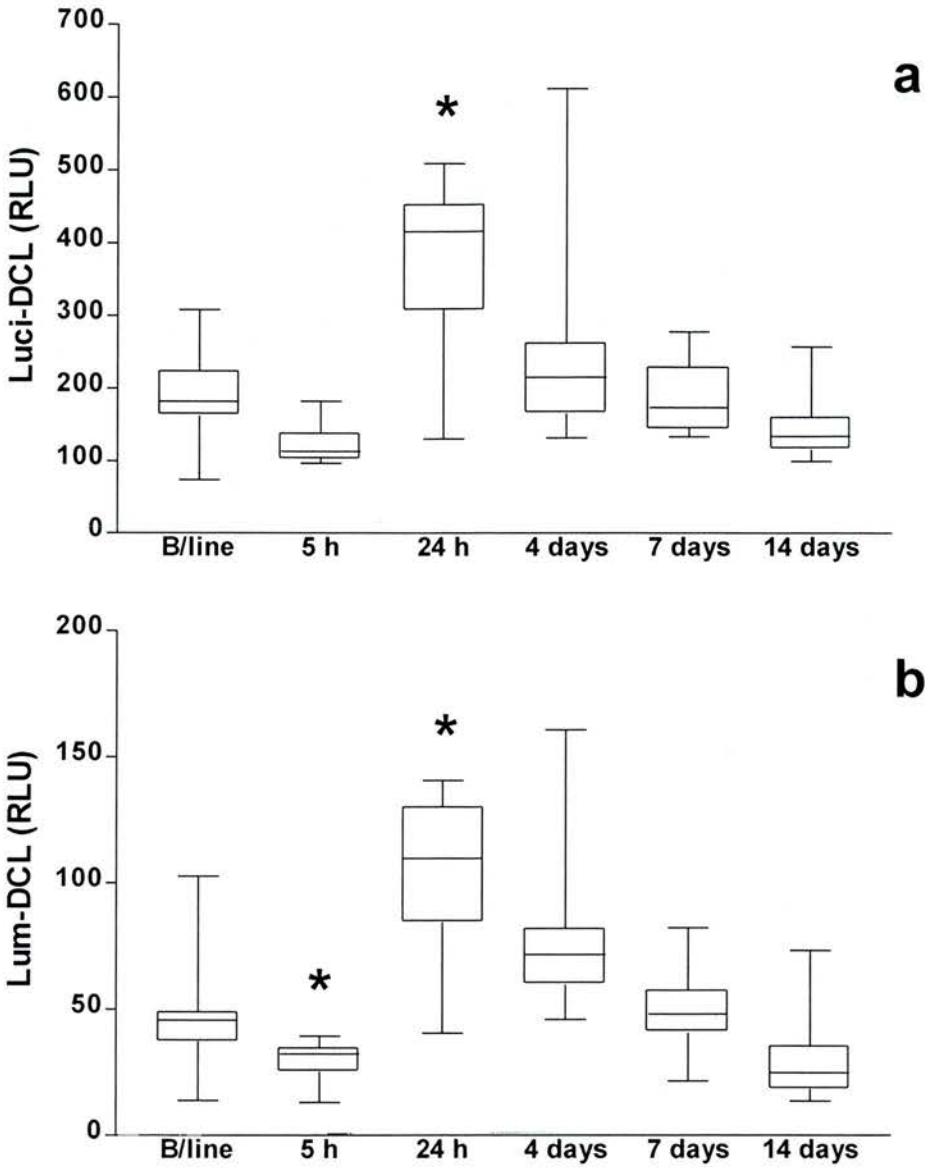


Figure 5.8a,b: Effect of hay/straw challenge on PMA-stimulated Luci- and Lum-DCL in peripheral blood neutrophils from COPD-susceptible animals

PMA-stimulated (100 ng/ml) Luci- and Lum-DCL of peripheral blood neutrophils isolated 10 days before and at the time points indicated after challenge were measured over a 90 min time course. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

a: PMA-stimulated Luci-DCL; note scale (0-700 RLU)

b: PMA-stimulated Lum-DCL; note scale (0-200 RLU)

reflects the difficulty in recruiting animals with a readily managed disease for a long-term study such as this, whilst they still have a useful working life.

The aim of the hay/straw challenge was to induce clinical signs of lung disease of sufficient severity to significantly increase clinical scores (score ≥ 3) and BALF neutrophil numbers without compromising the horses' welfare. Indeed, although clinical scores were significantly increased at 5 h, 24 h and 4 days after challenge, at no time did any of the animals become overtly distressed.

The most common presenting clinical signs reported in animals suffering from COPD that are examined in equine practice are chronic coughing, nasal discharge and poor exercise tolerance (McPherson and Thomson, 1983; Dixon *et al.*, 1995b; Ainsworth, 1999). Cough and nasal discharge are often intermittent signs, and as such were not regularly observed during the resting clinical examination at the post-challenge time points in this study. By contrast, increased respiratory effort or dyspnoea at rest was commonly observed after hay/straw challenge. The inflammation and diffuse obstruction of airways that occurs in affected animals leads to significant ventilation/perfusion mismatching resulting in a normocapnic hypoxaemia (Derksen *et al.*, 1988; West, 1992). This stimulates an increased respiratory effort that can be measured as an increased maximal change in intrapleural pressure (Robinson *et al.*, 1996). Previous work in this laboratory suggested that the dyspnoea detected clinically during the standard examination protocol employed correlated well with independently measured, increased maximal intrapleural pressure changes (Dixon *et al.*, 1995c). After challenge, abnormal respiratory sounds were frequently apparent on auscultation over both the lung fields and the cervical trachea and their detection was enhanced during induced hyperpnoea. In a recent prospective study of horses with naturally occurring COPD presented to a Veterinary hospital (Naylor *et al.*, 1992), abnormal lung sounds were detected at rest in 69% of cases, but after inducing hyperpnoea with a rebreathing bag this increased to 88%.

In the present study, clinical scores remained significantly elevated for at least 4 days after a 5 h challenge, although by this time pulmonary gas exchange had improved significantly, as evidenced by recovery from the arterial hypoxaemia detected at 5 and 24 h. Early studies of COPD-affected animals (Sasse, 1971; McPherson *et al.*,

1978) stressed the importance of a decreased PaO_2 (<85 mmHg) and an increased maximal intrapleural change as prerequisites for the conclusive diagnosis of the disease. However, more recent studies incorporating diagnostic respiratory cytology have indicated that many clinical cases of COPD would not be identified by these stringent diagnostic criteria (Mair, 1987a; Dixon *et al.*, 1995c). Although PaO_2 fell below 85 mmHg in only 2/6 animals after challenge, all horses developed arterial hypoxaemia relative to baseline values suggesting that hay/straw challenge significantly impaired pulmonary gas exchange in COPD-susceptible horses. The response of COPD-susceptible horses to this challenge system is consistent with previous work in this laboratory, where lung dysfunction (documented by measurement of pulmonary mechanics) occurred within 90 min and arterial hypoxaemia by 5 h. Furthermore, hay/straw challenge did not induce lung dysfunction in normal horses (McGorum *et al.*, 1993d).

The trachea of a normal horse should contain no visible secretions (Dixon, 1997) and the presence of more than a few flecks (grade 1; Table 2.5) is a sensitive indicator of pulmonary disease (Whitwell and Greet, 1984; Dixon *et al.*, 1995c). However, even normal horses will have small accumulations of secretions following periods of housing under conventional (hay/straw) management conditions (Buechner-Maxwell *et al.*, 1996). Thus baseline endoscopic scoring of secretions (Section 5.2.5) was consistent with the horses being in disease remission. In some horses, the volume of tracheal secretions did remain elevated (>grade 1) beyond 24 h, when this increase ceased to be significant for the whole group. This finding is consistent with our own observations (A. Kemmish, T.J. Brazil and S.D. Carrington, unpublished observations) and those of Jeffcoat *et al.* (1998), indicating that some horses with COPD have significantly elevated levels of immunologically-detectable respiratory mucins for long (2-3 weeks) periods after the resolution of clinical signs and BALF neutrophilia. However, the period of 8 weeks between the test challenge and the start of the experimental protocol was considered to be adequate for the resolution of any significant pathology related to the previous challenge.

Information on the effect of hay/straw challenge on peripheral blood leucocyte counts in COPD-susceptible horses is scant and highly variable (Derksen *et al.*, 1985b; Fairbairn *et al.*, 1993; Watson *et al.*, 1997; Marr *et al.*, 1998a). In general,

haematological indices have been considered to be of little value in the diagnosis of clinical cases of COPD (McPherson *et al.*, 1978; Beech, 1991). The current study is the first to monitor horses sequentially during and after an acute episode of induced COPD and to document a consistent increase in total leucocyte and neutrophil counts after challenge. Derksen *et al.* (1985b) found no change in peripheral leucocyte counts in either COPD-susceptible or control animals sampled following 3-7 days of continuous hay/straw challenge. Watson *et al.* (1997) identified a peripheral leucocytosis and neutrophilia in one of 5 affected horses after 12–14 days of hay/straw challenge, whereas Olszewski and Laber (1993) reported a consistent leucocytosis compared to control animals in horses with COPD of an undefined duration. Fairbairn *et al.* (1993) observed a small yet significant increase in peripheral blood neutrophil numbers at the end of a 7 h hay/straw challenge, but the neutrophilia had returned to baseline levels by 24 h. However, subsequent studies from the same laboratory have identified decreases in total leucocyte, neutrophil, eosinophil and monocyte counts after 5-7 h of challenge (Marr *et al.*, 1997a; Marr *et al.*, 1998a). In the current study, the leucocytosis present at 24 h was accounted for entirely by an increase in the absolute number of neutrophils (Figure 5.5b), probably due to mobilisation of neutrophils from the bone marrow storage pool into the circulating granulocyte pool in response to inflammation (Boggs, 1967; Lassen and Swardson, 1995). Release of marrow granulocytes occurs within hours of the onset of an inflammatory response in both man (Boggs, 1967) and horses (Lassen and Swardson, 1995) and relatively immature forms enter the circulation within 5 h of the onset of lung inflammation in rabbits (Lawrence *et al.*, 1996) or following antigen inhalation challenge in allergic dogs (Wood *et al.*, 1998).

In the face of an inflammatory response, total leucocyte and neutrophil counts in peripheral blood are thought to reflect a balance between an increase in the size of the marginated neutrophil pool and recruitment of cells to the inflammatory focus on the one hand and the rate of outflow of neutrophils from the bone marrow on the other (Boggs, 1967; Lassen and Swardson, 1995). This may account for the maintenance of leucocyte numbers at baseline levels at 5 h after challenge (Figure 5.5b). At 24 h post-challenge, the reduction in BALF neutrophil cell counts from the peak at 5 h (see Figure 6.1) suggests that recruitment of neutrophils to the lung has

ceased, by which time bone marrow production of neutrophils may exceed tissue demand and the rate of margination and allow a neutrophilia to develop (Carakostas *et al.*, 1981; Lassen and Swardson, 1995). A similar peak in peripheral blood neutrophil counts associated with significant outflow of cells from the bone marrow and an increase in the production of progenitor cells in the bone marrow has been documented in dogs allergic to *Ascaris suum*, 24 h after antigen inhalation challenge (Wood *et al.*, 1998).

In the horse (Carakostas *et al.*, 1981), as in man (Kronkite and Vincent, 1960), the half-life of neutrophils in the circulating pool is increased during an inflammatory response. This occurs in spite of the massive recruitment of neutrophils to the inflammatory focus (Kronkite and Vincent, 1960). This may reflect release of "younger" cells from the bone marrow, with a greater potential longevity as suggested by the slower rate of constitutive apoptosis in neutrophils collected 24 h after hay/straw challenge (Figure 5.6). Indeed, recent data from a model of streptococcal pneumonia in rabbits demonstrated that although neutrophils recently released from the bone marrow marginate and sequester normally in the pulmonary vasculature, they migrate into infected tissue more slowly than "older" neutrophils (Sato *et al.*, 1998). Thus a hypothesis can be formulated that if recently released cells are less susceptible to apoptosis and as neutrophils do not undergo apoptosis in the circulation, as previously marginated "younger" cells re-enter the circulating pool, the mean rate of apoptosis of neutrophils in a peripheral blood sample would decrease.

However, neutrophil apoptosis may also be inhibited by inflammatory mediators (Lee *et al.*, 1993) such as cytokines, either circulating or generated locally during an inflammatory response (Ussov *et al.*, 1996; Keel *et al.*, 1997). Identification of delayed rates of apoptosis in peripheral blood neutrophils harvested from human patients with severe sepsis (Keel *et al.*, 1997), severe burns (Chitnis *et al.*, 1996), systemic inflammatory response syndrome and following major elective surgery (Marshall and Watson, 1997) and from mice treated with thioglycollate (Coxon *et al.*, 1996), would suggest that exposure of neutrophils to circulating inflammatory mediators is critically important in regulating rates of neutrophil apoptosis in inflammatory diseases. Moreover, plasma from patients with severe burns and

systemic inflammatory response syndrome can inhibit apoptosis in neutrophils from healthy donors (Chitnis *et al.*, 1996; Marshall and Watson, 1997). Also a neutralising antibody to human GM-CSF abrogated the anti-apoptotic effect of the burns patients' plasma, confirming a role for circulating cytokines such as GM-CSF in the regulation of neutrophil lifespan *in vivo*. Very little is known about circulating inflammatory mediators in horses with COPD since few studies have addressed this question directly. Plasma thromboxane B_2 (Gray *et al.*, 1989), serotonin (Eyre, 1972) and 15-HETE (Gray *et al.*, 1992a) and urine LTE_4 (Doucet *et al.*, 1991) are elevated in affected horses following hay/straw challenge. The effects of serotonin and thromboxane B_2 on neutrophil apoptosis are unknown. 15-HETE has no effect on human neutrophil apoptosis *in vitro* (Hebert *et al.*, 1996) but despite LTB_4 affording dose-dependent inhibition in human neutrophils, this eicosanoid had no effect on equine neutrophil apoptosis (Section 4.2.2). Although platelets do not appear to play any direct role in the pulmonary response to hay/straw challenge (Fairbairn *et al.*, 1993), peripheral blood platelet responsiveness is enhanced (Ablett *et al.*, 1997) and as demonstrated in human neutrophils *in vitro* (Cox and Radford, 1999), soluble factors released from platelets may be important in regulating neutrophil lifespan *in vivo*.

Therefore, both of the above factors, namely an increased circulating number of relatively immature neutrophils and the systemic release of inflammatory mediators, may contribute to the observed inhibition of peripheral blood neutrophil apoptosis following challenge.

BALF collection alone has been reported to induce increases in absolute peripheral blood neutrophil numbers in humans (Von Essen *et al.*, 1991), dogs (Cohen and Batra, 1980) and monkeys (Cohen and Batra, 1980; Krombach *et al.*, 1985; Haley *et al.*, 1989). Also peripheral blood neutrophil PMA-stimulated Luci-DCL was significantly enhanced 24 h after a single BAL procedure in cynomolgus monkeys (Krombach *et al.*, 1985). The fever and flu-like symptoms experienced by 30% of human patients after BAL has been associated with increased circulating levels of TNF-α, IL-6 and IL-1β (Krause *et al.*, 1997). Although such cytokine assays were not available in the current study, the repetitive sham BAL procedure had no effect on peripheral blood neutrophil numbers or neutrophil CL activity (Section 5.2.2).

This suggests that horses are more resilient to the insult of BAL than some other species and that changes in blood neutrophil number and function observed in horses after hay/straw challenge would not be confounded by an effect of the BAL procedure itself.

It is well established that the presence of increased numbers of neutrophils in the circulation and even within the airspaces does not in itself necessarily imply causation of any ongoing lung injury and the functional status of these cells is a more critical factor (Martin *et al.*, 1989; Pittet *et al.*, 1997). However, only a few studies have addressed the functional status of peripheral blood neutrophils in horses affected with COPD. The phagocytic capacity of neutrophils from COPD-affected horses was reported to be unchanged compared to control animals (Nuytten *et al.*, 1983; Klucinski *et al.*, 1994). The increased fMLP-stimulated CL response of peripheral blood neutrophils after hay/straw challenge in the current study is the first observation of *in vivo* neutrophil priming in the horse. This *in vivo* response is consistent with the paradigm of priming in equine neutrophils as defined in the preceding *in vitro* studies (see Figure 3.17). Hence the stimulus of hay/straw challenge appears to prime circulating neutrophils, permitting functional coupling of fMLP receptors, but does not itself stimulate a significant respiratory burst in these cells. In a recent report (Olszewski *et al.*, 1999) however, peripheral blood neutrophils isolated from two COPD-affected horses after an undefined period of hay/straw challenge showed significantly enhanced serum-treated zymosan-stimulated superoxide anion generation in comparison to four control horses. Although superoxide generation in neutrophils from the affected horses was only 40% greater than in those from controls, this data does suggest that neutrophils were primed *in vivo*. This is in contrast to other reports of peripheral blood neutrophil respiratory burst activity in COPD-affected horses (Olszewski and Laber, 1993; Marr *et al.*, 1997a). In an experimental study, Marr and co-workers (1997) found no change in agonist (PAF, serum treated zymosan and PMA)- stimulated superoxide anion generation 5 and 24 h after the start of a 7 h challenge. However, they did find a small but significant increase in basal (unstimulated) superoxide anion generation at 24 h. Olszewski and Laber (1993) found no difference in basal respiratory burst activity, measured by the nitroblue tetrazolium method, between clinical COPD

cases and control horses. The increase in basal superoxide anion production following a controlled challenge (Marr *et al.*, 1997a) is interesting in light of the enhanced basal CL response over a 5 min time course detected in the current study, 24 h after challenge (see Figure 5.7a). These workers measured superoxide anion generation over two time courses (15 and 30 min) but did not report the kinetics of the enhanced response. However, given that the increment in basal respiratory burst activity in both the current study and that reported by Marr *et al.* (1997) was very small (Luci-DCL: 0.5 RLU, superoxide anion generation: 3.9 nmol/10^6 cells, respectively) the biological significance of this observation remains unclear. Increases in spontaneous (unstimulated), as well as agonist-stimulated, neutrophil respiratory burst activity have been reported in human patients with severe inflammatory disorders such as ARDS (Zimmerman *et al.*, 1983; Chollet-Martin *et al.*, 1992) and after major trauma (Botha *et al.*, 1995). However, such endogenous intravascular activation is likely to be associated with significant tissue injury and organ failure (Botha *et al.*, 1995).

Although spontaneous neutrophil activation has been reported in the circulation of asthmatics following antigen challenge (Durham *et al.*, 1984; Carroll *et al.*, 1985), this was assessed by increased C3b receptor expression, and hence is more likely to represent a priming related phenomenon (Condliffe *et al.*, 1996). Basal respiratory burst activity in human asthmatics is not increased following antigen challenge (Sustiel *et al.*, 1989). However, a number of studies in symptomatic asthma patients have demonstrated significant neutrophil priming and the authors suggested that the fMLP-stimulated response was a more sensitive index of *in vivo* priming than the response to non-physiological agonists such as PMA (Meltzer *et al.*, 1989; Sustiel *et al.*, 1989). Sustiel *et al.* (1989) also observed that not only were neutrophils primed but they were also less sensitive to downregulatory signals such as adenosine and prostaglandin E_2. Although this was not confirmed in a similar study (Meltzer *et al.*, 1989), it is worthy of note given the reduced airway mucosal prostaglandin E_2 production in horses with COPD (Gray *et al.*, 1992b).

It is interesting to note that peripheral blood neutrophil priming in horses following a single hay/straw challenge (Figures 5.7b) is shortlived, similar to that observed in human patients with a high risk status for multi-organ failure following severe torso

trauma. Botha and co-workers (1995) monitored fMLP-stimulated superoxide anion generation over the first 72 h after trauma. Primed neutrophils were detected in the circulation by 3-6 h but neutrophil function had returned to normal levels by 24-48 h. Furthermore, *in vivo* neutrophil priming appeared to be maximal in these patients (response was not further enhanced by subsequent PAF priming *in vitro*). This suggests that although the functional status of circulating neutrophils is a risk factor for neutrophil sequestration and tissue injury, *in vivo* priming *per se* does not reliably predict either the severity or outcome of injury.

The response of blood neutrophils to PMA has been widely used to assess changes in neutrophil function in humans (Zimmerman *et al.*, 1983; Chollet-Martin *et al.*, 1992) and in animal models (Watts *et al.*, 1990; Marr *et al.*, 1997a) of lung inflammation. Although priming of PMA-stimulated Luci-DCL in equine neutrophils did not occur *in vitro* (see Section 3.2.3.6) and antigen challenge had no effect on PMA-stimulated superoxide anion generation in a previous study (Marr *et al.*, 1997a), PMA-stimulated CL was significantly increased *in vivo* at 24 h with the current hay/straw challenge system (Figure 5.8a,b). Similar increases in PMA-stimulated neutrophil respiratory burst activity have been documented in many pulmonary inflammatory conditions in humans including ARDS (Zimmerman *et al.*, 1983; Chollet-Martin *et al.*, 1992), bacteraemia (Bass *et al.*, 1986) and asthma (Meltzer *et al.*, 1989). The reduction in PMA-stimulated Lum-DCL in equine neutrophils 5 h after challenge (Figure 5.8b) was initially somewhat perplexing, however a reduction in yeast-stimulated peripheral blood neutrophil Lum-DCL has been reported in human subjects 5 h after inhalation of LPS (Michel *et al.*, 1997). The authors of this latter study speculated that this might reflect sequestration of a primed subpopulation of neutrophils in the pulmonary vasculature at this early time point. Indeed, this contention may have some basis as flow cytometric studies of blood neutrophils harvested from human patients with ARDS (Chollet-Martin *et al.*, 1992) and bacteraemia (Bass *et al.*, 1986) suggested that the increased net PMA-stimulated H_20_2 production detected was mostly associated with a hyperresponsive sub-population of cells. Furthermore, priming slows neutrophil transit time through (Ussov *et al.*, 1996), and increases sequestration within, the pulmonary vasculature (Worthen *et al.*, 1987) due to changes in cell size and stiffness (Worthen *et al.*, 1989;

Downey *et al.*, 1990). *In vivo* priming of human neutrophils has been documented by light microscopic assessment of cell shape change *ex vivo* (Ussov *et al.*, 1996). Although not formally assessed in this study, shape-changed neutrophils were rarely recognized during many haemocytometer counts of isolated cells after challenge. However, functional priming of equine neutrophils *in vitro* did not invariably result in significant cell shape change (see Section 3.2.3.3).

Recently, Marr and colleagues (1997b) have extended their earlier work demonstrating CD18-dependent and agonist-enhanced (PAF, hrIL-8 and hrC5a) adherence of equine neutrophils to fibronectin-coated plastic (Foster *et al.*, 1997; Marr *et al.*, 1999). They showed that unstimulated neutrophil adherence to fibronectin-coated plastic was significantly increased in a CD18-dependent fashion at 24 h after challenge and remained elevated at 72 h but had returned to pre-challenge levels by 1 week. The most interesting finding however was the lack of enhanced adherence at 5 h post-challenge and the authors suggested that the early neutrophil recruitment to the lung was independent of increased integrin function. These data are consistent with neutrophil priming leading to enhanced integrin-mediated adhesion (Bochsler *et al.*, 1990; Worthen *et al.*, 1992; Condliffe *et al.*, 1998b). However the absence of functionally upregulated neutrophils in the circulation at 5 h may, as discussed earlier, reflect enhanced retention of primed cells within the pulmonary vasculature at this early time point when recruitment to the lung is maximal (Fairbairn *et al.*, 1993).

Another factor that may play a role in the enhanced respiratory burst activity of peripheral blood neutrophils after challenge is the enhanced potential for oxidant generation in relatively immature neutrophils freshly released from the bone marrow. Data from a rabbit model of bacteraemia indicate that during an inflammatory response neutrophils are released from the bone marrow faster than they mature (Sato *et al.*, 1998). Although immature neutrophils have lower chemotactic and phagocytic capacities than mature circulating cells (Altman and Stossel, 1974), cytoplasmic granule size and number are increased. Primary or azurophil granules form at an early stage in neutrophil development, reducing in size and number with subsequent mitoses (Bainton, 1988). Hence, early release of neutrophils with more granules, containing myeloperoxidase and other enzymes, may enhance the

respiratory burst capacity and destructive capability of relatively immature cells (Terashima *et al.*, 1996). This may be of particular relevance in the generation of luminol (myeloperoxidase)-dependent CL.

The stimulus or stimuli responsible for priming peripheral blood neutrophils *in vivo* for enhanced respiratory burst activity following hay/straw challenge of COPD-susceptible horses is unknown. Marr and colleagues (1997) speculated, quite reasonably, that some "neutrophil activating factor(s)" or cytokine(s) was released into the circulation in response to antigen challenge as has been detected in the serum of human asthmatics in association with the early and late responses following antigen challenge (Durham *et al.*, 1984; Carroll *et al.*, 1985). Also transit of neutrophils through an inflamed site, in this case the lung, may be an important priming mechanism as a consequence of neutrophil contact with "non-released" cytokines and lipid mediators (such as PAF) expressed on the surface of activated endothelial cells (Zimmerman *et al.*, 1990).

This question has not as yet been addressed in equine COPD, but data from *in vitro* studies have implicated IL-8 and MIP-2 secreted by equine alveolar macrophages as possible mediators of the enhanced neutrophil chemotactic activity in BALF from affected horses (Franchini *et al.*, 1998). However, other likely candidates worthy of investigation include TNF-α, GM-CSF and IL-6 (Michel *et al.*, 1992; Clapp *et al.*, 1994; Pittet *et al.*, 1997). The preliminary attempts made in the current study to assess the potential roles of IL-8 and TNF-α are discussed in Section 6.2.4 and further collaborative work is ongoing in this laboratory to improve the currently available assays for equine IL-6, IL-8 and TNF-α.

CHAPTER 6

KINETICS, FUNCTION AND FATE OF AIRSPACE NEUTROPHILS IN COPD - SUSCEPTIBLE HORSES FOLLOWING HAY / STRAW CHALLENGE

6.1 INTRODUCTION

It is now quite clear that the clinical signs of exercise intolerance, airway obstruction and increased airway secretions recognized in horses suffering from COPD are intimately associated with a neutrophilic inflammatory response in the small airways (reviewed in Chapter 1). Although the numbers of neutrophils recovered from the airways are not correlated with the severity of clinical signs (Grunig *et al.*, 1989), post-mortem studies demonstrated a close association between the neutrophil content of BALF and the severity of histological changes in the lungs of affected horses (Viel, 1983).

Thus if the neutrophil's armoury of proteolytic enzymes, reactive oxygen species and highly cationic proteins were secreted to excess or inappropriately within the pulmonary interstitium or airspaces, this cell type may well be central to the pathogenesis of the structural and functional lung injury observed in equine COPD. The classical double-edged sword (Smith, 1994) of the neutrophil's microbicidal capability is well recognized in human medicine. The importance of neutrophils as mediators of host tissue injury is now well accepted in the pathogenesis of many inflammatory diseases that afflict humans (such as chronic bronchitis, emphysema, asthma, rheumatoid arthritis and reperfusion injury after myocardial infarction) and in clinically significant and potentially disabling disorders of the horse such as intestinal ischaemia/reperfusion injury, septic arthritis and COPD (reviewed in Sections 1.6 and 1.7). Decades of research in the field of human neutrophil biology have helped to define both the regulation of neutrophil function and many of the mediators implicated in this "inappropriate" neutrophil-driven, tissue injury.

Histopathological examination of pulmonary tissues from horses with longstanding and severe COPD suggested that significant irreversible structural lung damage was not a common feature of this disease (Kaup *et al.*, 1990a; Kaup *et al.*, 1990b). However, the development of more sensitive techniques to evaluate airway structure and function have documented increased small airway wall thickness (Broadstone *et al.*, 1997) and perhaps of more interest, alveolar epithelial damage inferred from enhanced rates of alveolar clearance (Votion *et al.*, 1998; Votion *et al.*, 1999). It is conceivable that such pathological changes may be caused by the release of pro-inflammatory and histotoxic neutrophil secretory products. Assessment of more subtle measures of lung function *in vivo*, such as alveolar epithelial cell permeability, may prove valuable in the investigation of the dynamic relationship between neutrophil influx and lung injury in equine COPD.

Although some studies have begun to define certain aspects of equine neutrophil function *in vitro* (discussed in Chapter 3), relatively few have investigated *in* or *ex vivo* neutrophil function in COPD (Nuytten *et al.*, 1983; Olszewski and Laber, 1993; Klucinski *et al.*, 1994; Marr *et al.*, 1997a; Marr *et al.*, 1997b; Olszewski *et al.*, 1999). Although the increased amounts of neutrophil-derived proteases found in the tracheal secretions of affected animals (Maisi *et al.*, 1994; Koivunen *et al.*, 1997a; Koivunen *et al.*, 1997b; Raulo and Maisi, 1998) offer circumstantial evidence for neutrophil degranulation *in vivo*, it is not possible from these data alone to conclude that this is occurring in the small airways.

Although scintigraphic (Fairbairn *et al.*, 1993) and bronchoalveolar fluid (McGorum *et al.*, 1993d) studies have reported the early kinetics of the pulmonary sequestration of neutrophils and their appearance in the airways (within 5 h), knowledge of the dynamics of airspace cell populations beyond the first 7 h are lacking.

The importance of cell priming in regulating both the functional responsiveness of neutrophils to secretagogue stimuli *in vitro*, and the severity of neutrophil-mediated injury *in vivo*, is now well established (Condliffe *et al.*, 1998b). Preliminary reports suggest that both peripheral blood neutrophil (Marr *et al.*, 1997a; Olszewski *et al.*, 1999) and airway phagocyte respiratory burst activity (Olszewski and Laber, 1993) are enhanced in equine COPD, but the kinetics of changes in the priming and

activation status of both blood and airspace neutrophils following controlled hay/straw challenge has not been previously studied.

The longstanding assumption that the fate of neutrophils recruited to an inflammatory focus was one of *in situ* necrosis and disintegration seemed somewhat at odds with clinical observations in some human diseases, such as lobar streptococcal pneumonia, which although characterized by influx of massive numbers of neutrophils, resolves completely and without residual lung injury (Haslett, 1992). An alternative to *in situ* necrosis with the inevitable leakage of the neutrophil's histotoxic constituents into the surrounding tissue was suggested by the seminal work of the 19th Century cell biologist, Elie Metchnikoff (Metchnikoff, 1893). In 1891, in a series of lectures delivered at the Pasteur Institute in Paris, Metchnikoff described vital microscopy studies in which he observed "englobement" of "microphages" (later termed "neutrophils") by resident macrophages within an inflamed focus. Almost a century later observation that ageing neutrophils underwent constitutive apoptosis, thereby facilitating their recognition and phagocytosis by local macrophages, formalised Metchnikoff's recognition of an alternative clearance pathway for effete and functionally redundant neutrophils (Newman *et al.* 1982; Savill *et al.* 1989).

Several features of the neutrophil apoptosis/macrophage clearance paradigm suggest that this represents an indispensable pathway in the successful resolution of inflammation without significant host tissue destruction and scarring. An individual macrophage was capable of recognizing apoptotic cells by several discrete mechanisms and could phagocytose and rapidly degrade several apoptotic neutrophils without inciting a pro-inflammatory response by the macrophage (reviewed in Section 1.5.4). Consequently it seemed likely that this process may also be important in equine COPD.

With the neutrophil established as a central player in the pulmonary inflammatory response that underlies equine COPD, the laboratory techniques and data that provided initial characterization of equine neutrophil priming, activation and apoptosis *in vitro* were harnessed to assess the functional status and role of these cells in an experimental hay/straw challenge model *in vivo*.

It was hoped that a better understanding of the functional consequences of hay/straw challenge on neutrophil function and kinetics in the airspaces of COPD-susceptible horses would facilitate progress towards elucidating the cellular pathogenesis of this disease and hence may assist in the development of novel therapeutic strategies.

6.2 RESULTS

6.2.1 EFFECT OF HAY/STRAW CHALLENGE ON THE KINETICS OF AIRSPACE NEUTROPHIL RECRUITMENT AND CLEARANCE

6.2.1.1 Effect of sham BAL protocol on BALF cell numbers and function

To ensure that serial BAL alone did not cause significant alterations in BALF absolute or differential cell counts or function, five sham exposure BAL collections were performed at 0 h, 19 h, 4, 7 and 14 days in 6 asymptomatic COPD-susceptible horses as described in Section 2.8.3 and Table 2.4.

At 0 h, median (range) recovery of BALF was 46.0% (38.0-63.3%) of the fluid instilled (n = 6). Recovery of BALF at all subsequent time points was not significantly different. Viability of cells in raw BALF (assessed by trypan blue exclusion) at 0 h was 96.6% (93.6-100%). Cell viability at all subsequent time points was not significantly different. The total nucleated and differential cell counts in BALF collected at each time point are shown in Table 6.1. Total nucleated, absolute and differential cell counts at the subsequent time points were not significantly different to those determined at 0 h.

The effect of these lavages on the respiratory burst activity of BALF cells was assessed in 3 horses. BALF cells were processed as described in Section 2.8.1 and PBS- and fMLP (1 μM)-stimulated Luci- and Lum-DCL were measured over a 5 min time course and PBS-, ZAP (10% v/v)- and PMA (100 ng/ml)-stimulated Luci- and Lum-DCL were measured over a 90 min time course as described in Section 2.8.4. Neither basal (PBS only) nor agonist-stimulated Luci- and Lum-DCL of BALF cells harvested at the subsequent sham BAL collection time points (19 h and 4, 7 and 14 days) was significantly different to that measured at 0 h (data not shown).

Cell type	0 h	19 h	4 days	7 days	14 days
Total cell count ($x 10^5$/ml)	6.8 (3.9-9.0)	3.8 (1.8-4.9)	3.7 (2.4-5.8)	4.9 (4.0-5.2)	7.2 (4.9-5.2)
Neutrophil (%)	2.2 (1.0-3.3)	3.0 (1.0-6.3)	4.2 (1.7-5.2)	1.1 (0.7-3.8)	1.5 (1.0-4.8)
Macrophage (%)	43.8 (32.0-55.2)	43.6 (27.6-50.3)	49.1 (31.0-50.8)	43.0 (29.1-52.5)	47.6 (42.9-52.9)
Lymphocyte (%)	53.8 (42.2-61.9)	49.7 (46.6-64.8)	41.4 (39.6-63.9)	49.0 (44.1-67.0)	45.5 (44.4-51.3)
Eosinophil (%)	0.3 (0.0-0.4)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)
Mast cell (%)	1.5 (0.0-2.4)	1.2 (0.0-4.7)	1.7 (0.0-6.4)	1.0 (0.0-3.2)	1.3 (0.3-2.1)
Basophiloid (%)	0.0 (0.0-0.1)	0.0 (0.0-0.4)	0.1 (0.0-1.2)	0.5 (0.0-2.8)	0.1 (0.0-0.9)
Epithelial (%)	0.3 (0.0-1.6)	0.1 (0.0-0.5)	0.6 (0.0-2.0)	0.0 (0.0-2.5)	0.3 (0.0-0.3)

Table 6.1: Total and differential cell counts in BALF collected from 6 asymptomatic COPD-susceptible horses during sham protocol

BALF was collected as described in Section 2.7.3 at the time points indicated. Total nucleated and differential cell counts [mean (range)] were performed in duplicate on raw BALF using a haemocytometer and on Leishman's-stained cytocentrifuge preparations, respectively, as described in Section 2.8.2. Total and differential cell counts did not differ throughout the sham protocol.

6.2.1.2 BALF cell kinetics following challenge

BALF was collected at least 10 days prior (baseline) and 5 h, 24 h and 4, 7 and 14 days after the start of the 5 h hay/straw challenge (Section 2.7.3 and Table 2.4). The volume of BALF recovered at baseline [44.5% (42.7-60.0%) of volume instilled] was not different to that recovered during the first sham collection. Likewise, BALF recovery was not significantly different from baseline at any of the post-challenge time points. Cell viability in the baseline BALF sample [97.4% (94.9-98.7%)] was not different to that in the first sham BALF sample and did not alter at any of the post-challenge time points (data not shown).

Total nucleated cell counts in BALF were significantly elevated 5 and 24 h after challenge, mirroring a massive influx (35-fold increase in median counts) of neutrophils that peaked at the 5 h time point, remained elevated at 24 h, but returned to baseline levels by 4 days (Table 6.2). Recruitment of neutrophils to the airspaces was of sufficient magnitude to elevate total BALF cell numbers in spite of a surprising but consistent net reduction in (i) the numbers of alveolar macrophages recovered at 5 and 24 h and (ii) the number of lymphocytes recovered at 24 h (Table 6.2). This suggests that there is considerable trafficking of airspace macrophages and lymphocytes as well as neutrophils after hay/straw challenge. Indeed, statistical analysis of the serial differential cell counts demonstrated a small but significant increase in the proportion of neutrophils in BALF at 4 days as well as 5 and 24 h (see Table 6.3 and Figure 6.1). This probably reflects the significant decrease in absolute macrophage numbers between 5 h and 7 days after challenge (at day 4 the reduction in macrophage numbers just failed to reach statistical significance, $p = 0.09$, see Figure 6.2). In fact in two horses with less striking BALF neutrophilia after challenge, differential neutrophil counts showed a modest increase between 5 and 24 h (median values; 14.3 - 20.6% and 21.9 - 22.6%) in spite of the consistent decline in absolute neutrophil numbers ($2.3 - 1.9 \times 10^5$/ml and $2.2 - 1.4 \times 10^5$/ml). This effect was likely to have been due to the reduced macrophage numbers at these time points. A small but significant increase in the proportion of mast cells was observed 4 days after challenge (Table 6.3).

Representative photomicrographs of BALF cells collected at baseline and 5 h after the start of the hay/straw challenge are shown in Figure 6.3a,b on page 214.

Cell type	B/line	5 h	24 h	4 days	7 days	14 days
Total ($x 10^5$/ml)	8.0 (4.9-13.1)	**14.2* (10.5-64.2)**	**6.8* (5.1-17.1)**	5.2 (3.8-7.9)	5.8 (2.5-6.6)	7.7 (4.6-11.1)
Neutrophil ($x 10^5$/ml)	0.2 (0.1-0.6)	**7.7* (2.3-57.7)**	**1.5* (1.1-12.0)**	0.3 (0.2-0.6)	0.2 (0.1-0.4)	0.2 (0.1-6.5)
Macrophage ($x 10^5$/ml)	3.7 (2.2-6.2)	**2.0* (1.7-3.4)**	**1.5* (1.4-2.8)**	2.1 (1.2-3.3)	**1.8* (1.5-2.6)**	3.4 (1.9-3.9)
Lymphocyte ($x 10^5$/ml)	4.1 (2.4-6.7)	3.8 (2.4-6.7)	**2.5* (1.7-3.6)**	2.3 (1.7-4.1)	3.1 (0.8-4.7)	3.9 (2.5-7.3)
Eosinophil ($x 10^5$/ml)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Mast cell ($x 10^5$/ml)	0.0 (0.0-0.3)	0.2 (0.1-0.3)	0.2 (0.0-0.4)	0.1 (0.0-0.3)	0.1 (0.1-0.2)	0.1 (0.0-0.2)
Basophiloid ($x 10^5$/ml)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.01 (0.0-0.2)	0.0 (0.0-0.1)	0.0 (0.0-0.0)
Epithelial ($x 10^5$/ml)	0.0 (0.0-0.1)	0.1 (0.0-0.1)	0.0 (0.0-0.1)	0.1 (0.0-0.1)	0.0 (0.0-0.4)	0.0 (0.0-0.1)

Table 6.2: Total and absolute cell counts in BALF collected from 6 COPD-susceptible horses following hay/straw challenge

BALF was collected as described in Section 2.7.3 at the time points indicated. Total nucleated and absolute cell counts [mean (range)] were performed in duplicate on raw BALF using a haemocytometer and calculated from differential cell counts of Leishman's-stained cytocentrifuge preparations, as described in Section 2.8.2. Values in bold text with an * are significantly different from baseline values for the same cell type ($p<0.05$). B/line: Baseline.

Cell type	B/line	5 h	24 h	4 days	7 days	14 days
Neutrophil (%)	2.2 (1.0-4.8)	**61.8* (14.3-89.9)**	**26.4* (20.6-70.0)**	**5.8* (4.1-11.5)**	2.4 (0.5-7.3)	2.8 (1.4-4.6)
Macrophage (%)	45.0 (32.3-52.9)	**14.2* (3.1-32.2)**	**23.8* (13.0-28.8)**	41.8 (30.3-47.5)	35.8 (26.4-59.5)	41.8 (31.7-47.8)
Lymphocyte (%)	50.4 (44.4-63.3)	22.6 (6.7-64.6)	**43.3* (15.3-54.4)**	47.2 (43.3-58.1)	60.4 (45.8-66.8)	52.8 (47.8-65.3)
Eosinophil (%)	0.3 (0.0-0.2)	0.0 (0.0-0.8)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.5)
Mast cell (%)	0.8 (0.1-2.1)	0.6 (0.2-1.1)	2.7 (0.0-4.6)	**1.7* (0.4-3.4)**	1.8 (0.0-5.6)	1.4 (0.4-2.2)
Basophiloid (%)	0.2 (0.0-0.9)	0.2 (0.0-0.9)	0.0 (0.0-0.2)	0.3 (0.0-2.4)	0.0 (0.0-2.4)	0.2 (0.0-1.6)
Epithelial (%)	0.02 (0-0.07)	0.05 (0-0.11)	0.04 (0.02-0.09)	0.08 (0-0.14)	0.02 (0.01-0.35)	0.01 (0-0.06)

Table 6.3: Differential cell counts in BALF from 6 COPD-susceptible horses following hay/straw challenge

BALF was collected as described in Section 2.8.3 at the time points indicated. Differential cell counts [mean (range)] were performed in duplicate on Leishman's-stained cytocentrifuge preparations of raw BALF as described in Section 2.9.2. Values in bold text with an * are significantly different from baseline values for the same cell type ($p<0.05$). B/line: Baseline.

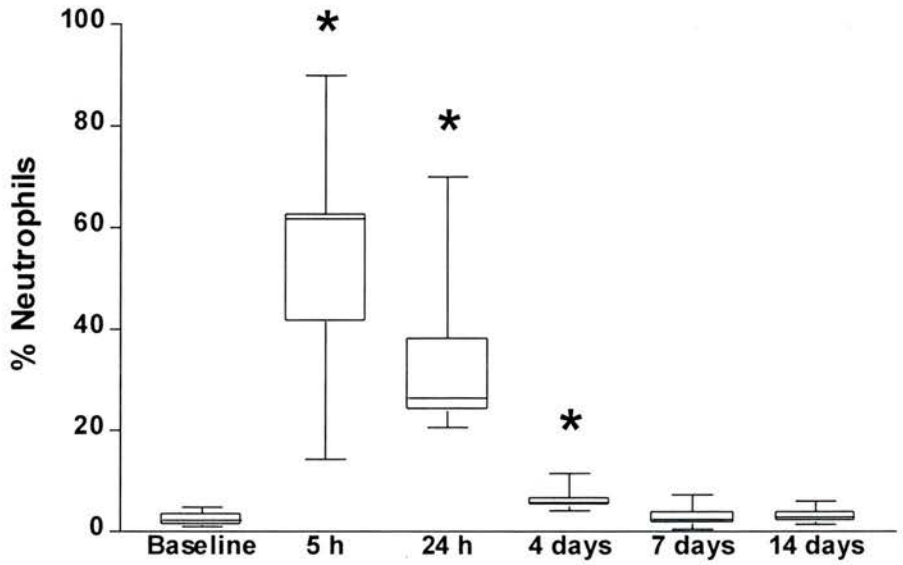


Figure 6.1: Kinetics of BALF neutrophil recruitment and clearance following hay/straw challenge of 6 COPD-susceptible horses; differential cell counts

BALF was collected (as described in Section 2.7.3) 10 days before (baseline) and at the time points indicated after challenge. Differential cell counts of raw BALF were performed in duplicate on Leishman's-stained cytocentrifuge preparations. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, $n = 6$)

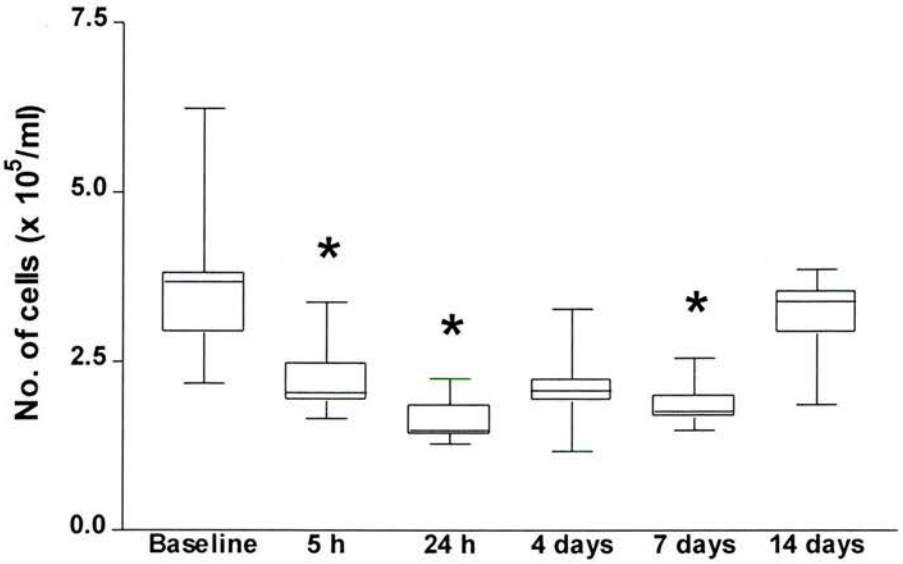


Figure 6.2: Kinetics of BALF macrophage numbers following hay/straw challenge of 6 COPD-susceptible horses; absolute cell counts

BALF was collected (as described in Section 2.7.3) 10 days before (baseline) and at the time points indicated after challenge. Absolute cell counts were performed in duplicate on raw BALF using a haemocytometer and macrophage numbers (plotted on y-axis) calculated from differential cell counts of Leishman's-stained cytocentrifuge preparations. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. ($*$; $p<0.05$ compared to baseline values, $n = 6$)

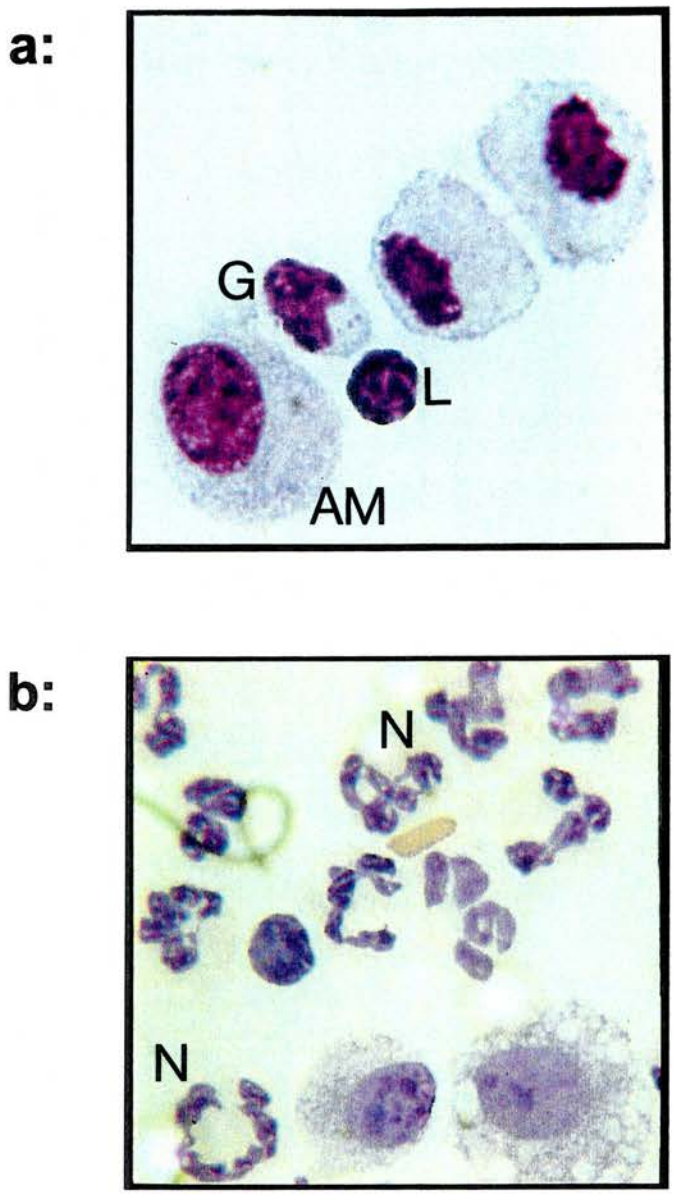


Figure 6.3a,b: Cytological features of BALF cell populations before and 5 h after hay/straw challenge of COPD-susceptible horse

BALF was collected from COPD-susceptible horses 10 days before and immediately after a 5 h hay/straw challenge. Leishman's-stained cytocentrifuge preparations were examined under oil immersion. Representative photomicrographs of BALF cells at baseline and 5 h are shown.

a: baseline: Alveolar macrophages (**AM**) and lymphocytes (**L**) predominate. An atypical or granular lymphocyte is also present (**G**). Magnification x 1320

b: 5 h: Neutrophils (**N**) are the predominant cell type. Magnification x 1280

6.2.2 EFFECT OF CHALLENGE ON BALF CELL CHEMILUMINESCENCE

Chemiluminescence of BALF cells harvested from 6 asymptomatic COPD-susceptible horses at least 10 days prior (baseline) and 5 h, 24 h and 4, 7, and 14 days after a 5 h hay/straw challenge was measured in the presence of lucigenin (Luci-DCL) and luminol (Lum-DCL) in response to PBS (Basal), fMLP (1 μM), ZAP (10% v/v) or PMA (100 ng/ml) as described in Section 2.8.4.

6.2.2.1 Basal chemiluminescence

The basal (PBS, unstimulated) Luci- and Lum-DCL of BALF cells recovered following hay/straw challenge (all time points) was not statistically different to the CL activity of BALF cells obtained prior to challenge (baseline) whether recorded over a 5 min or a 90 min time course ($p>0.05$, $n = 6$, data not shown).
However, scrutiny of the individual horse data does highlight some noteworthy findings.

(i) There was little change in basal BALF cell Luci-DCL after challenge compared to baseline responses.

(ii) In three horses, basal luminol (myeloperoxidase and therefore neutrophil specific, see Section 2.8.3.4)-DCL of BALF cells was markedly enhanced compared to baseline activity (approximately 100 fold) at 5 and 24 h after challenge when neutrophil numbers in their BALF were increased. However, the variability between individual horses precluded this effect from reaching significance for the group as a whole.

(iii) Two of the three horses whose basal BALF cell Lum-DCL did not increase greatly after challenge were the two animals with the smallest increases in differential BALF neutrophil count (baseline and 5 h neutrophil proportions: 4.8% and 21.9%; 3.1% and 14.3%).

These data demonstrate the considerable heterogeneity amongst different COPD-susceptible horses in their response to hay/straw challenge.

6.2.2.2 fMLP-stimulated chemiluminescence

fMLP-stimulated Luci-DCL (Figure 6.4a) and Lum-DCL (Figure 6.4b) of BALF cells was greatly enhanced at 5 and 24 h after challenge. Although the increase in the fMLP-stimulated Luci-DCL response at 5 h was not statistically significant, scrutiny of the raw data indicated that this was due to an inexplicably low CL response in only one horse, in which the CL response was less than at baseline. However, ZAP-stimulated Luci-DCL of BALF cells from this animal was also extremely low but fMLP- and ZAP-stimulated Lum-DCL responses were of a similar magnitude to the rest of the group. Thus it is likely that this anomaly was due to experimental error rather than an inherent defect in the respiratory burst mechanisms of this animal's cells.

Scrutiny of the fMLP-stimulated Luci-DCL data indicate that other cell types in BALF, most probably alveolar macrophages, contribute to fMLP-stimulated Luci-DCL (Figure 6.4a, baseline data), consistent with studies of fMLP-stimulated superoxide anion generation in adherent human alveolar macrophages (Holian and Daniele, 1979). Although the Luci-DCL response of BALF cells at baseline was small in comparison to that observed at 5 and 24 h when neutrophils were the predominant cell type, it is not possible to assume that recruited neutrophils alone were the source of the additional CL activity. Alveolar macrophage respiratory burst activity is also likely to be enhanced after challenge as reported in human asthmatic patients in comparison to normal control subjects (Calhoun and Bush, 1990) and in ovalbumin sensitised guinea pigs following antigen challenge (Okada *et al.*, 1993). The fMLP-stimulated Lum-DCL response in BALF cells was markedly enhanced at 5 and 24 h in parallel with the number of neutrophils present (see Figures 6.4 and 6.1).

6.2.2.3 PMA - and ZAP -stimulated Chemiluminescence

PMA-stimulated Luci-DCL was significantly enhanced 24 h after challenge (Figure 6.5a). The median PMA-stimulated Luci-DCL of BALF cells was greatly enhanced at 5h, but, like the fMLP-stimulated Luci-DCL data, due to considerable inter-animal variation did not reach statistical significance ($p = 0.09$). The response of BALF cells to PMA would also suggest that alveolar macrophage respiratory burst activity

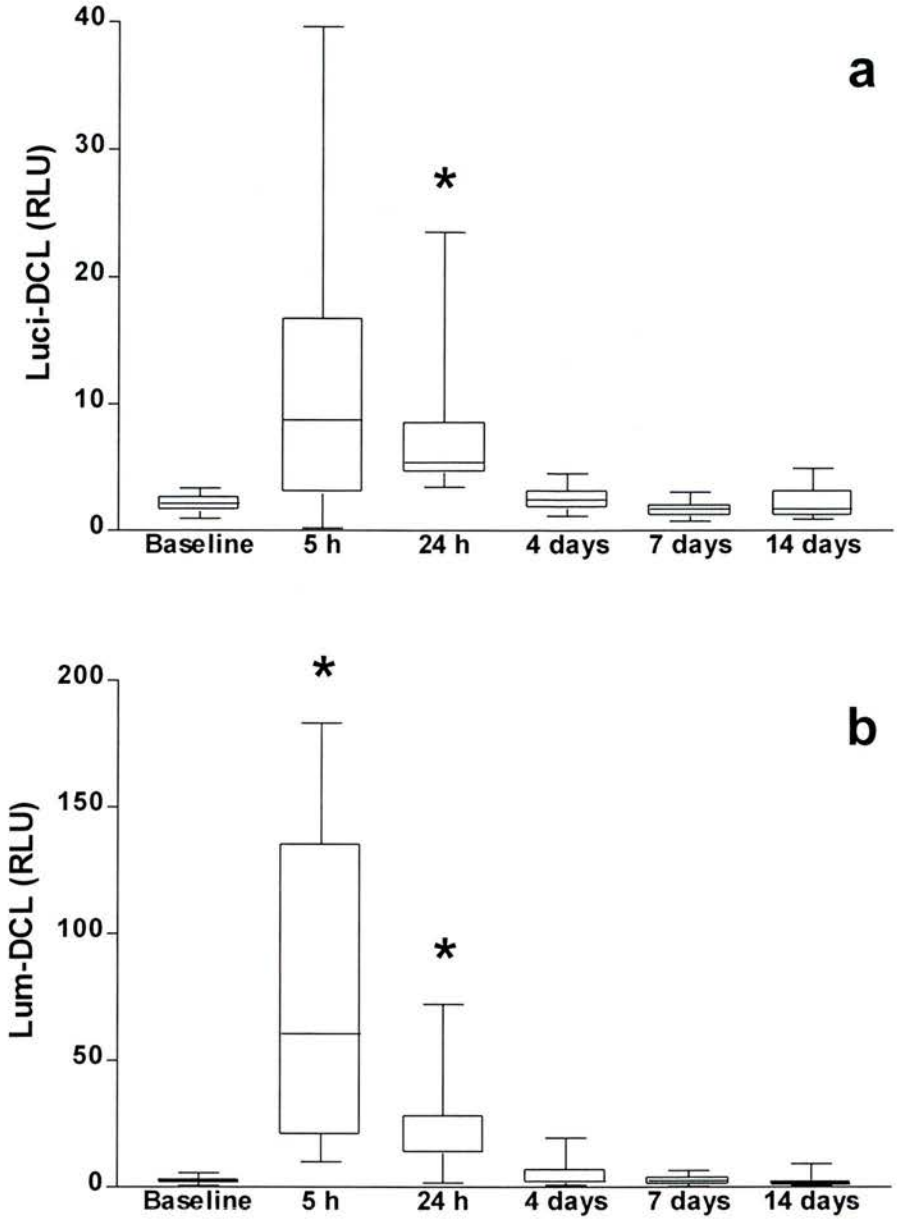


Figure 6.4a,b: Effect of hay/straw challenge on fMLP-stimulated Luci-DCL and Lum-DCL of BALF cells collected from COPD–susceptible horses

fMLP-stimulated (1 μM) Luci- and Lum-DCL of BALF cells harvested 10 days before (baseline) and at the time points indicated after challenge were measured over a 5 min time course. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (*; $p<0.05$ compared to baseline values, n = 6)

a: fMLP-stimulated Luci-DCL; note scale (0-40 RLU)

b: fMLP-stimulated Lum-DCL; note scale (0-200 RLU)

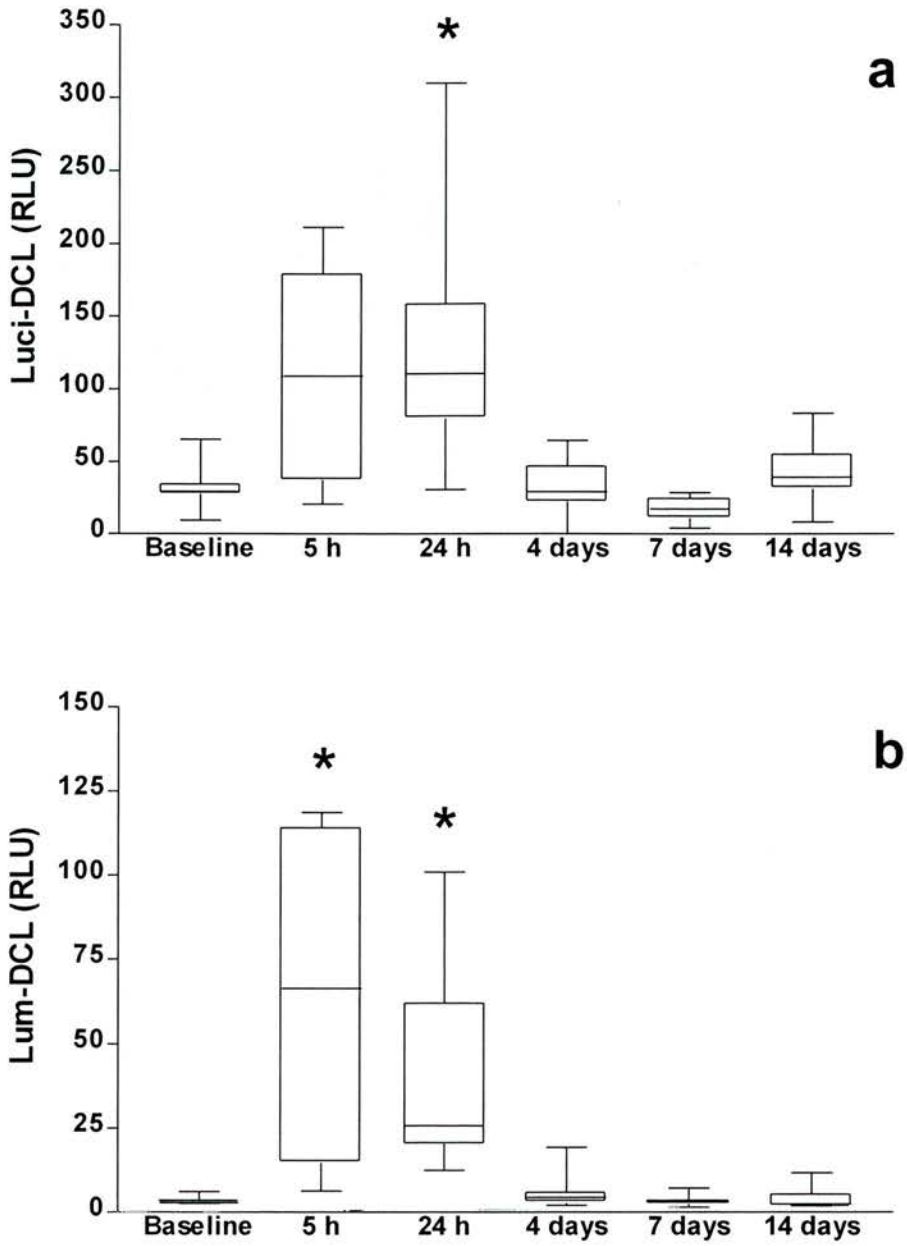


Figure 6.5a,b: Effect of hay/straw challenge on PMA-stimulated Luci- and Lum-DCL of BALF cells collected from COPD–susceptible horses

PMA-stimulated (100 ng/ml) Luci- and Lum-DCL of BALF cells harvested 10 days before (baseline) and at the time points indicated after challenge were measured over a 90 min time course. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

a: PMA-stimulated Luci-DCL

b: PMA-stimulated Lum-DCL

was upregulated after challenge because the maximal CL response of BALF cells was recorded at 24 h in 4/6 horses when BALF neutrophil numbers had fallen from their peak at 5 h. Moreover, it is highly unlikely that the PMA responsiveness of the residual neutrophils at 24 h was significantly greater than that of the neutrophils present at 5 h. PMA-stimulated Lum-DCL was increased at both 5 and 24 h (Figure 6.5b), following a similar pattern to the fMLP- and ZAP-stimulated Lum-DCL responses.

Simple comparison of the magnitudes of the Lum-DCL responses of BALF cells at 5 and 24 h after challenge with that of quiescent peripheral blood neutrophils (Figure 3.4b) would suggest that neutrophils recruited to the airspaces of COPD-susceptible horses after hay/straw challenge are primed for an enhanced respiratory burst in response to PMA.

Although individual variation was again apparent (see Section 6.2.2.2), there was little difference in ZAP-stimulated Luci-DCL of BALF cells following hay/straw challenge (median 54.3 RLU; range, 13.8-116.2 RLU omitting data from the horse with extremely low response at 5 h) in comparison to baseline responses (86.0; 14.5-115.2, $p>0.05$). As ZAP-stimulated Luci-DCL generation (qualitatively comparable with superoxide anion generation) at baseline was of a similar magnitude to that measured after challenge (when neutrophils make up a significant proportion of the mixed BALF cell population studied) a number of conclusions could be drawn. Firstly, cell types normally resident in the airspaces when horses are in clinical remission, most probably alveolar macrophages, have a similar capacity to generate superoxide anions in response to ZAP as neutrophils recruited to the airspaces following challenge. Alternatively, the inhaled challenge stimulus may have complex and differential effects on the superoxide anion generating capacity of different cell types, which could only be dissected following the successful separation of individual cell types into populations of high purity. Finally, a desensitisation of the response of recruited neutrophils to C5a with concurrent enhancement of alveolar macrophage activity seems unlikely in light of the major ZAP-stimulated Lum-DCL response (again mirroring neutrophil numbers) of the same mixed BALF cell populations (Figure 6.6). A lack of comparative literature on the ZAP-stimulated oxidative activity of BALF cells leaves this question unresolved.

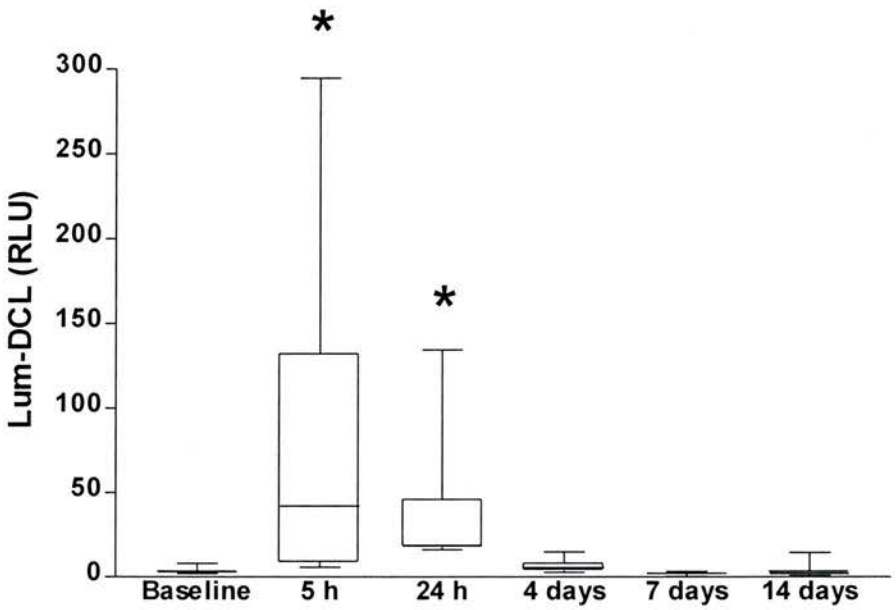


Figure 6.6: Effect of hay/straw challenge on ZAP-stimulated Lum-DCL of BALF cells collected from COPD–susceptible horses

ZAP-stimulated (10% v/v) Lum-DCL of BALF cells harvested 10 days before and at the time points indicated after challenge was measured over a 90 min time course. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

6.2.2.4 Comparison of peripheral blood and BALF neutrophil respiratory burst activity

The CL data presented in Chapter 5 confirm that peripheral blood neutrophils harvested from COPD-susceptible horses are primed following hay/straw challenge. Furthermore, the data presented above (Sections 6.2.2.1-6.2.2.3) indicate that the respiratory burst activity of BALF cells is greatly enhanced after challenge; BALF cytology (Section 6.2.1.2) and Lum-DCL responses would suggest that the latter effect is largely due to the recruitment of large numbers of neutrophils into the airways.

However, from these data alone it is not possible to compare directly the functional status of peripheral blood and airspace neutrophils following challenge. This represented a key question in this study and due to the difficulties in isolating pure populations of neutrophils from BALF (Section 2.8.3) cannot be answered with certainty. However, the highly neutrophil-specific CL signal generated in the presence of luminol (Section 2.8.3.4) did offer an opportunity to begin to address this question. Using differential cell count data (Table 6.3), fMLP-stimulated Lum-DCL data was normalised to produce values of Lum-DCL equivalent to that generated by 1×10^6 BALF neutrophils. These normalised data were compared to the fMLP-stimulated Lum-DCL response of 1×10^6 peripheral blood neutrophils at each time point using the Mann Whitney U test for unpaired data. The results of these analyses demonstrated that the fMLP-stimulated Lum-DCL response of neutrophils recruited to the airspaces was significantly greater than that of peripheral blood neutrophils at baseline, 5 h, 7 days and 14 days after challenge (Figure 6.7). Notably, analysis of data at 24 h and 4 days after challenge showed no such difference in the responses of the two populations of cells (Figure 6.7). These results suggest that for airspace neutrophils, priming is an integral part of their recruitment to the lung and transmigration into the airways. Comparison of data from the baseline, 7 and 14 day time points (when BALF neutrophils numbers were low) with responses at 5 h (when BALF neutrophilia was at its peak) supports this hypothesis. The difference in magnitude of the Lum-DCL responses of 1×10^6 blood and airspace neutrophils at these time points was similar, irrespective of the numbers of neutrophils recruited to the lung and by implication, the magnitude of the recruitment stimulus.

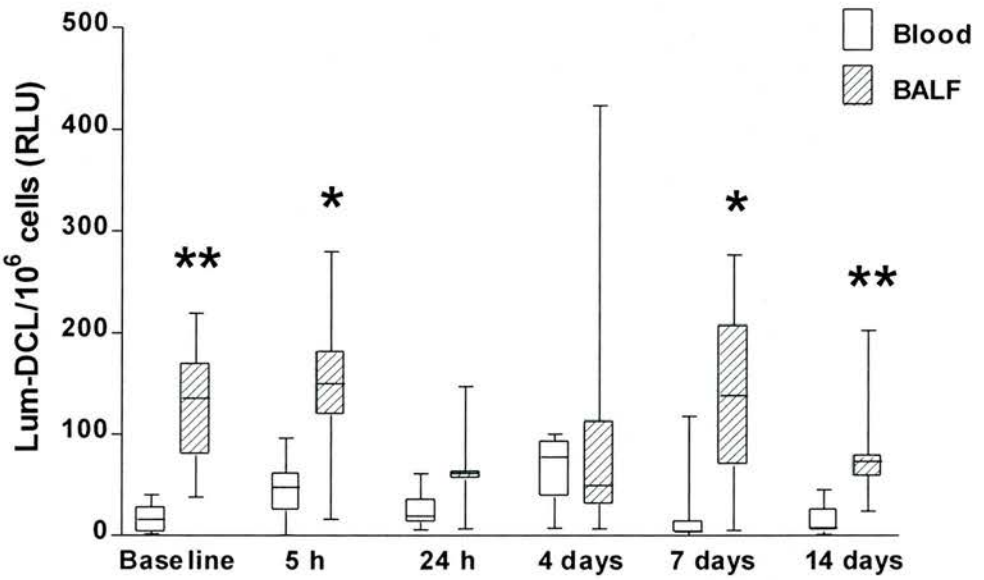


Figure 6.7: Comparison of fMLP-stimulated Lum-DCL of peripheral blood and BALF neutrophils from COPD-susceptible horses

fMLP-stimulated (1 μM) Lum-DCL of peripheral blood neutrophils (open boxes) and BALF cells (hatched boxes) harvested 10 days before (baseline) and at the time points indicated after challenge was measured over a 5 min time course. The Lum-DCL values for BALF neutrophils are the normalised data for the equivalent CL generation by 1 x 10^6 neutrophils. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (**; $p<0.01$, *; $p<0.05$ compared to values for peripheral blood neutrophils, n = 6)

The similarity in the Lum-DCL response of peripheral blood and BALF neutrophils at 24 h and 4 days is intriguing. Firstly the fMLP-stimulated Lum-DCL response of peripheral blood neutrophils was significantly increased in the whole group at 4 days and in 4/6 horses at 24 h (although this was not significant; Section 5.2.8.2). However of greater interest is the apparent reduction in the Lum-DCL activity of BALF (1×10^6) neutrophils at these time points. Neutrophil numbers in the lung had declined substantially by 24 h suggesting that the rate of recruitment had already decreased significantly. Consequently the reduced fMLP-stimulated Lum-DCL response of BALF neutrophils at 24 h and 4 days most likely reflects a downregulation in the functional status of the population of neutrophils that have yet to be cleared from the airspaces.

It must be recognized however, that the integrity of the derived data on which these conclusions are based is heavily reliant on the strength of the relationship between the proportion of neutrophils in BALF and the luminol-dependent CL signal (Section 2.8.3.4 and Figure 2.8a,b). Although this relationship was very robust in the *in vitro* experiments ($r^2 = 0.81$, $p<0.0001$, Figure 2.8a), the correlation was weaker when the *in vivo* challenge data were analysed ($r = 0.76$, $p<0.0001$, Figure 2.8b). The kinetics of the BALF neutrophilia induced by hay/straw challenge over the 14 day study period meant that the data were highly skewed, with only 8/36 BALF samples containing more than 25% neutrophils. This relationship should be re-examined using a larger number of BALF samples with a more balanced range of neutrophil proportions before this technique of assessing BALF neutrophil respiratory burst activity can be adopted with confidence. However, bearing in mind the constraints of the failure to isolate pure populations of neutrophils from BALF, this method does allow an initial appreciation of the comparative function of peripheral blood and BALF neutrophils harvested from COPD-susceptible horses before and after hay/straw challenge.

6.2.3 EFFECT OF CHALLENGE ON NEUTROPHIL ELASTASE CONCENTRATION AND ACTIVITY IN BALF

The concentration of ENE 2A in the supernatant of BALF collected 10 days before and at the routine time points after hay/straw challenge was measured using a specific ELISA (Section 2.8.5). The concentration of ENE 2A in BALF supernatant was significantly greater than baseline at 5 h, 24 h and 4 days after challenge (Figure 6.8), indicating that neutrophil degranulation occurred following recruitment to the airspaces. Of particular note was the continued presence of significant amounts of ENE 2A in BALF at 4 days (Figure 6.8) when neutrophil numbers had returned to baseline levels (Table 6.2). Peak concentrations of ENE 2A (55.3 ng/ml, 4.9-360.3; median and range) and peak neutrophil numbers in BALF were detected concurrently at 5 h after challenge. There was a strong correlation between the concentration of ENE 2A in BALF supernatant and the absolute numbers of neutrophils in whole BALF ($r = 0.74$, $p < 0.0001$). Also there was a significant correlation between the concentration of ENE 2A in the BALF supernatant and the basal (unstimulated) Lum-DCL of BALF cells ($r = 0.65$, $p < 0.0001$) suggesting that an increase in the spontaneous release of oxidants and extracellular proteinase secretion are closely associated. This evidence of degranulation of neutrophils recruited to the airspaces following challenge is further supported by a recent collaborative study undertaken in this laboratory investigating the role of matrix metalloproteinases in equine COPD (P. Maisi [University of Helsinki] and T.J. Brazil, unpublished observations). These studies have demonstrated significantly increased concentrations of MMP-9 (of which neutrophils are a major source [Clegg *et al.*, 1997a]), in BALF supernatant following hay/straw challenge and MMP-9 levels followed almost identical kinetics to that of ENE 2A described above.

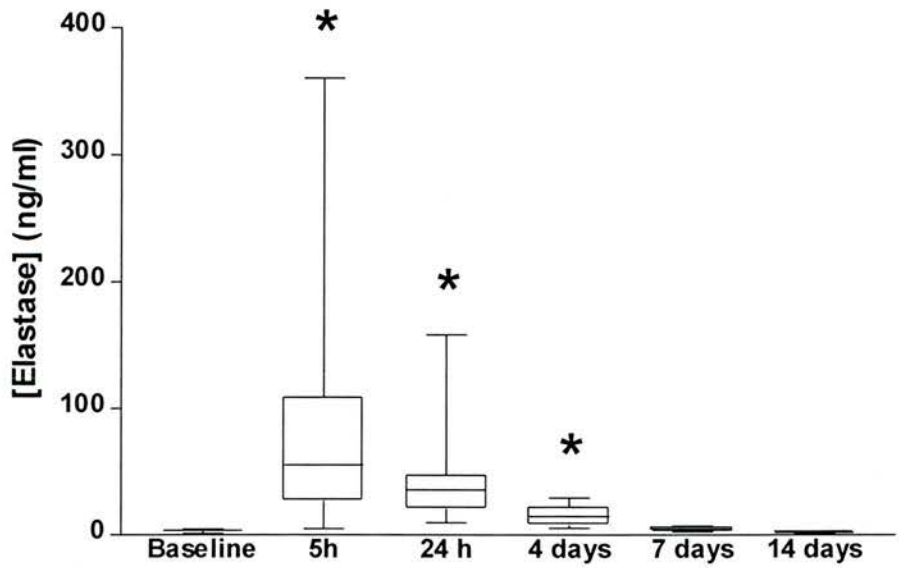


Figure 6.8: Effect of hay/straw challenge on equine neutrophil elastase 2A content of BALF from COPD-susceptible horses

The ENE 2A content in BALF supernatant harvested 10 days before (baseline) and at the time points indicated after challenge was measured by specific ELISA using purified ENE 2A as a standard. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

The presence of increased concentrations of immunologically detectable ENE 2A in BALF does not however, necessarily infer any pathogenetic role for elastase in COPD. Therefore, functional elastase activity was assayed in BALF supernatant using the specific substrate N-methoxysuccinyl-ALA-ALA-PRO-VAL p-nitroanilide (Section 2.8.6). Elastase activity was not detectable in any of the BALF supernatant samples collected either before or after challenge, even when incubated in the presence of an excess of the chromogenic substrate for up to 18 h in an attempt to detect very low levels of elastase activity. The change in absorbance and hence activity of the lowest concentration (3.2 ng/ml) of purified ENE 2A remained linear over this period (data not shown). These data would suggest that although neutrophil proteinases are released into the airspaces following challenge their appropriate antiproteinase inhibitors rapidly inactivate them.

6.2.4 EFFECT OF CHALLENGE ON CYTOKINE LEVELS IN BALF

The concentration of equine IL-8 in BALF supernatant harvested before and 5 h, 24 h and 4 days after challenge was measured by a dot blot assay, using erIL-8 as a reference standard, as described in Section 2.8.10. Although there was much inter-animal variation at all the time points studied, levels of IL-8 in BALF were significantly increased at 5 h after challenge (Figure 6.9). However, despite the only statistical difference in IL-8 concentrations being the increase between baseline and 5 h, a distinct pattern was noticed for all 6 horses. IL-8 levels increased at 5 h then fell markedly at 24 h before increasing again at 4 days (Figure 6.8) suggesting biphasic IL-8 production within the airways following hay/straw challenge. Analysis of cytokine levels in the same BALF samples using the specific human TNF-α and IL-8 and ovine IL-8 ELISA systems failed to demonstrate any cross-reactivity indicating that the Mabs employed did not recognize epitopes on the equivalent equine cytokines.

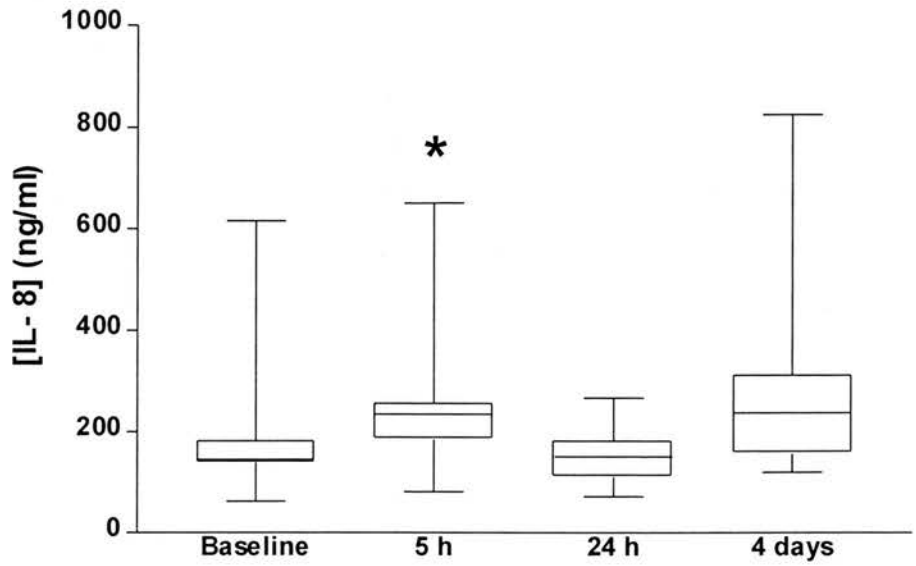


Figure 6.9: Effect of hay/straw challenge on levels of IL-8 in BALF from COPD-susceptible horses

The concentration of IL-8 in BALF supernatant harvested 10 days before (baseline) and at the time points indicated after challenge was measured by specific dot blot assay using purified erIL-8 as a standard (Section 2.8.10). Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

6.2.5 FATE OF NEUTROPHILS IN THE RESOLUTION OF AIRWAY INFLAMMATION FOLLOWING CHALLENGE

Cytological examination of BALF collected from COPD-susceptible horses confirmed that there was a rapid influx of neutrophils into the airspaces in response to hay/straw challenge (Figure 6.1). However, the kinetics of clearance of the recruited neutrophils and in particular their fate were central questions in the investigation of the resolution of airway inflammatory response. Figure 6.1 and Table 6.2 show that neutrophils are cleared rapidly from the airspaces after termination of the challenge stimulus. By 24 h, absolute neutrophil numbers had already fallen to a median of only 27.8% (12.8-67.7%) of their peak at 5 h (end of hay/straw challenge period) and had returned to baseline levels by 4 days. Cytological and immunohistochemical examinations of BALF, harvested before and after challenge, were performed to investigate the potential role of apoptosis of airspace neutrophils and whether phagocytosis by alveolar macrophages was involved in the clearance of the pulmonary neutrophil burden following hay/straw challenge.

6.2.5.1 Kinetics of airspace neutrophil apoptosis following challenge

The number of neutrophils with typical features of apoptosis was counted on Diff Quik-stained cytocentrifuge preparations; this staining technique gave the best definition of this morphological phenotype under oil immersion light microscopy (Figure 6.10a). Attempts to enumerate free apoptotic neutrophils on cytocentrifuge preparations stained by the TUNEL method proved unrewarding. The rigours of cell fixation and the labelling procedure appeared to cause significant disruption of neutrophil morphology and a high level of non-specific labelling making accurate enumeration of cells impossible. On Diff Quik-stained preparations, small numbers of apoptotic neutrophils were identified at all time points, even prior to challenge, but this number was significantly greater than baseline values at 24 h and 4 days after challenge (Figure 6.11a). The proportion (%) of the neutrophils present in BALF that had typical light microscopic features of apoptosis was significantly increased 4 days after challenge (Figure 6.11b).

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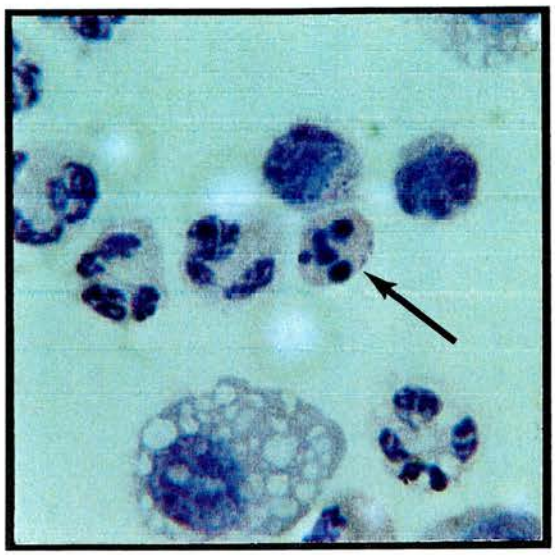

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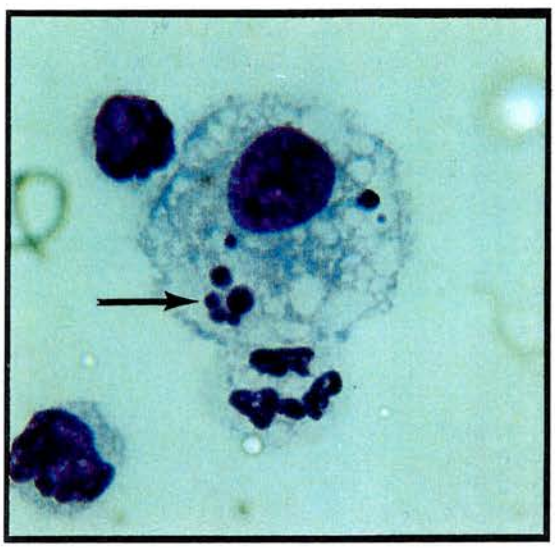

Figure 6.10a,b: Airspace neutrophils undergo apoptosis and are phagocytosed by alveolar macrophages *in vivo*; light microscopic evidence

BALF was collected 24 h after hay/straw challenge and the light microscopic morphology of neutrophils and alveolar macrophages examined on Diff Quik-stained cytocentrifuge preparations under oil immersion. Representative photomicrographs are shown.

a: Free apoptotic neutrophil in BALF (**arrow**); note densely staining pyknotic chromatin fragments. Magnification x 1240

b: Alveolar macrophage containing apoptotic neutrophil (**arrow**). Magnification x 1300

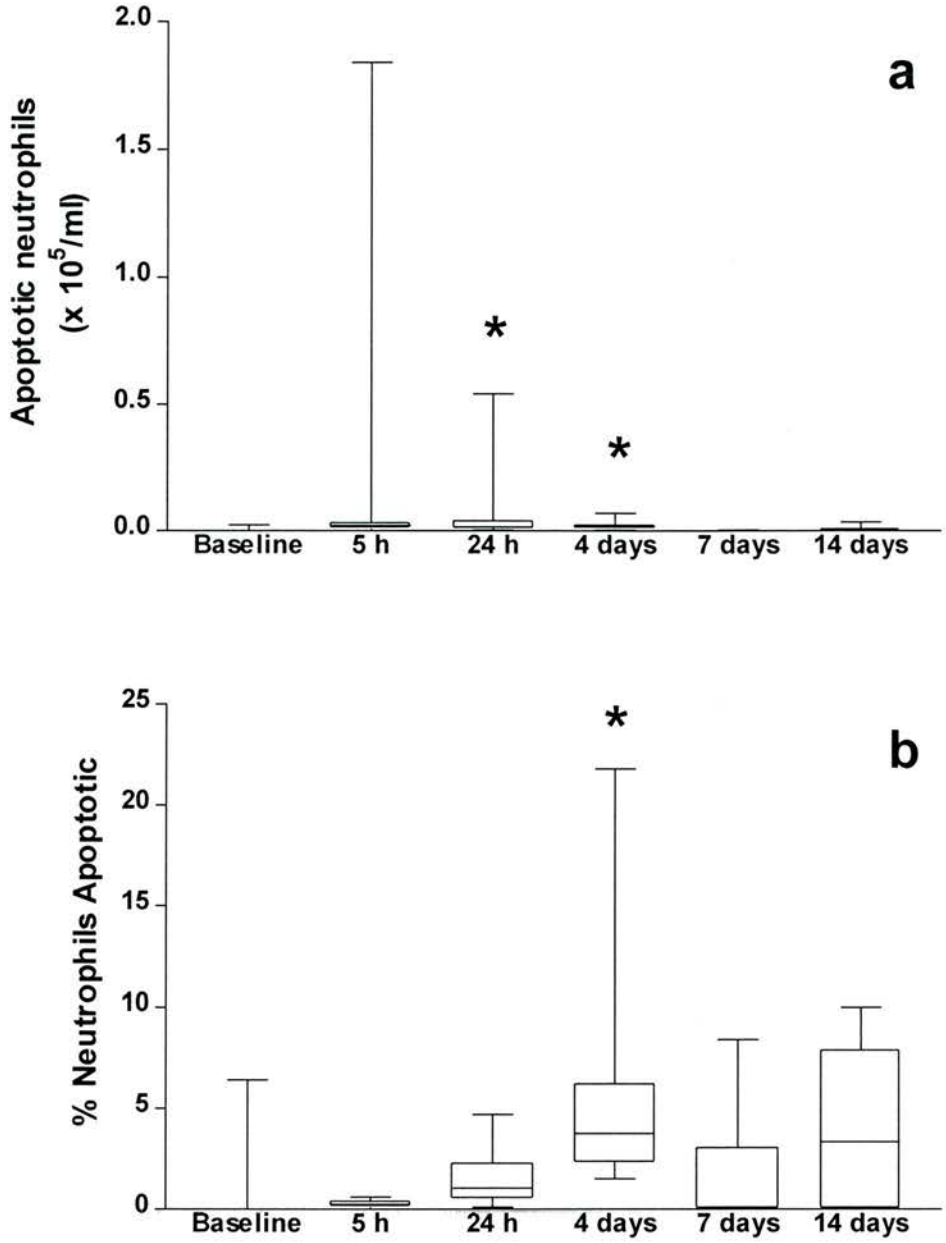

Figure 6.11a,b: Kinetics of airspace neutrophil apoptosis *in vivo*

BALF was collected 10 days before (baseline) and at the time points indicated after challenge. Neutrophils with typical light microscopic features of apoptosis were counted on duplicate Diff Quik-stained cytocentrifuge preparations. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (*; $p<0.05$ compared to baseline values, n = 6)

a: Absolute number of apoptotic neutrophils in BALF

b: Percentage of neutrophils in BALF with morphological features of apoptosis

6.2.5.2 Qualitative morphological evidence for macrophage clearance of apoptotic neutrophils *in vivo*

Light microscopic examination of Diff Quik-stained cytocentrifuge preparations of BALF clearly identified a small sub-population of alveolar macrophages that contained densely staining intracytoplasmic chromatin remnants consistent with these macrophages having phagocytosed apoptotic neutrophils *in vivo*; (Figure 6.10b). The appearance of these remnants varied from being clearly identified as an intact apoptotic neutrophil to a cluster of densely staining bodies to a tight group of tiny specks. The range in morphology of these remnants suggested that there was a continuum in macrophage processing of these effete cells *in vivo*, from recent ingestion of an intact (apoptotic) cell to complete degradation. Alveolar macrophages identified as having phagocytosed apoptotic chromatin contained either a single apoptotic cell or group of apoptotic bodies or up to 5 discrete apoptotic cells or clusters of pyknotic chromatin. To ensure that what appeared to be pyknotic chromatin on Diff Quik-stained preparations did indeed constitute apoptotic DNA, representative cytocentrifuge preparations of BALF collected 24 h after challenge were fixed and stained by TUNEL as described in Sections 2.6.6 and 2.8.8. TUNEL staining clearly identified clusters of apoptotic DNA within the cytoplasm of alveolar macrophages (Figure 6.12a). Typically this was recognized as tightly grouped clusters of apoptotic bodies. Although non-specific labelling was prominent in some preparations, in general, alveolar macrophages appeared to be more resilient to the processing required for TUNEL than neutrophils.

To confirm that this apoptotic DNA was of neutrophil origin, cytocentrifuge preparations were fixed and stained immunohistochemically specifically for equine neutrophil elastase 2A as described in Section 2.8.7. Counterstaining with Mayer's Haematoxylin clearly demonstrated that the intensely stained neutrophil elastase co-localised with the densely staining condensed nuclear chromatin within the cytoplasm of alveolar macrophages (Figure 6.12b). The pattern of ENE 2A staining of this ingested material complimented that recognized on Diff Quik-stained slides. Hence, some alveolar macrophages appeared to contain intact apoptotic neutrophils, others contained a small cluster of apoptotic bodies surrounded by intense ENE 2A staining, whilst in some cells no nuclear material could be identified but a

a:

b:

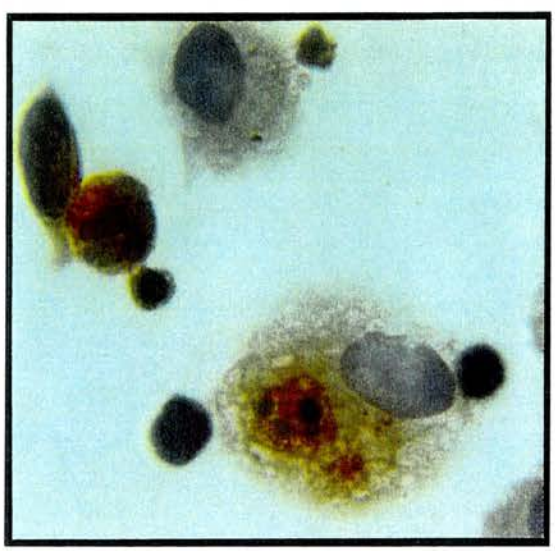

Figure 6.12a,b: Alveolar macrophages ingest apoptotic neutrophils *in vivo*; immunohistochemical evidence

BALF was collected 24 h after challenge and fixed and stained by the TUNEL method (Sections 2.6.6 and 2.8.8, **Panel a**) and immunohistochemically for ENE 2A (Section 2.9.7, **Panel b**) and examined under oil immersion. Representative photomicrographs are shown. **a**: Alveolar macrophage containing several clusters of TUNEL +ve apoptotic bodies. TUNEL labeling detected with DAB, slides counterstained with Methyl green, Magnification x 1850 **b**: Alveolar macrophage containing densely staining pyknotic bodies co-localised with ENE 2A (red). ENE 2A detected with AEC, slides counterstained with Mayer's Haematoxylin, Magnification x 1330

well-circumscribed cluster of granular ENE 2A staining remained. These patterns of ENE 2A staining are very similar to those of neutrophil myeloperoxidase identified in BALF macrophages from babies with neonatal respiratory distress syndrome (Grigg *et al.*, 1991). In the current study, individual ENE 2A positive alveolar macrophages demonstrated snapshots of a process that appeared to progress from engulfment of an intact apoptotic neutrophil to complete degradation of its component parts. These morphological features suggested that elastase remained immunologically detectable for some time after the processing and complete destruction of visible nuclear chromatin. However, from this limited number of observations undertaken in the clearance phase of the induced pulmonary neutrophilia, it is still not possible to determine whether all alveolar macrophages containing neutrophil remnants had initially engulfed an intact apoptotic cell. Alveolar macrophages may also have scavenged remnants of neutrophils that had undergone apoptosis and broken up into several smaller apoptotic bodies *in vivo*. Furthermore, neutrophil elastase either secreted by functionally competent neutrophils or liberated following secondary necrosis of apoptotic neutrophils *in vivo* may similarly have been scavenged by alveolar macrophages.

Further confirmatory evidence of alveolar macrophage clearance of effete neutrophils was sought from ultrastructural examination of BALF cells collected from COPD-susceptible horses 24 h after the start of the 5 h hay/straw challenge. BALF was collected and prepared for TEM as described in Sections 2.3.2.1 and 2.8.9. With careful examination of TEM grids, small numbers of both free apoptotic neutrophils and alveolar macrophages that had ingested intact apoptotic neutrophils could be identified (Figure 6.13a,b).

These investigations indicate that alveolar macrophage engulfment of apoptotic neutrophils play at least some part in the clearance of the neutrophil burden from the airspaces during the recovery phase of the acute pulmonary neutrophilia induced in COPD-susceptible horses by hay/straw challenge.

6.2.5.3 Kinetics of macrophage clearance of apoptotic neutrophils *in vivo*

Having confirmed that macrophage phagocytosis of effete neutrophils contributed to the resolution of the pulmonary neutrophil burden, the kinetics of this process were

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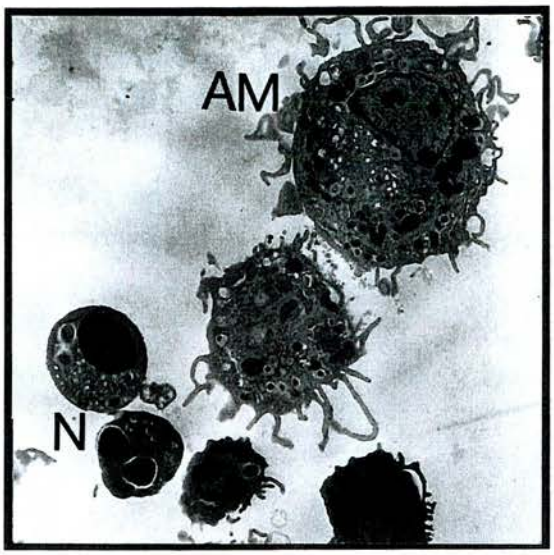

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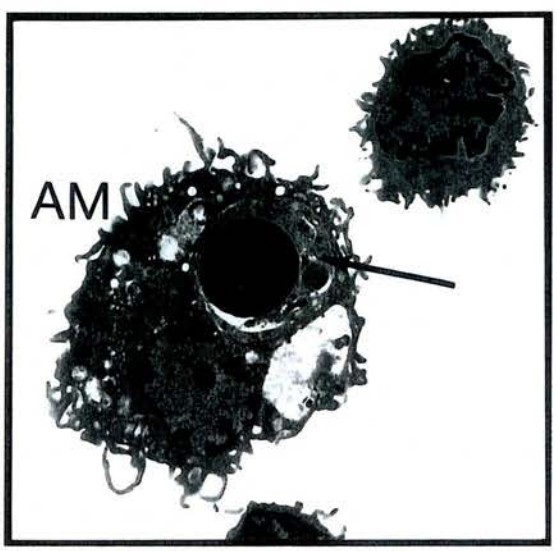

Figure 6.13a,b: Alveolar macrophages ingest apoptotic neutrophils *in vivo*; ultrastructural evidence

BALF was collected 24 h after hay/straw challenge and washed, fixed, prepared, examined and imaged as described in Section 2.3.2.1. Representative electron photomicrographs are shown.

a: Alveolar macrophage (AM) and two apoptotic neutrophils (N) "free" in BALF are shown. Note condensed nuclear chromatin and retention of intracellular granules in neutrophils. Magnification x 3730

b: Alveolar macrophage (AM) that has engulfed an intact apoptotic neutrophil (arrow). Magnification x 4800

examined by counting the number of alveolar macrophage that contained ENE 2A positive cell remnants at each time point. Even prior to challenge a small proportion of macrophages (0.5%, 0.3-0.9%, Figure 6.14) contained ENE 2A positive cell remnants indicating that macrophage clearance may represent a normal, physiological disposal pathway for neutrophils reaching the end of their functional life in the airways. The number of ENE 2A positive macrophages rose to a peak at 24 h (Figure 6.14), coinciding with the period of most rapid decline in BALF neutrophil numbers (Figure 6.1), and remained significantly elevated at 4 days when BALF neutrophil numbers had fallen to pre-challenge levels (Figure 6.14).
The temporal relationships between the percentages of neutrophils, apoptotic neutrophils and ENE 2A positive macrophages counted on appropriately stained cytocentrifuge preparations of BALF cells are shown in Figure 6.15a,b. Although not strictly valid for data that is not normally distributed, the data in Figure 6.15a,b are presented as means ± SEM in order to demonstrate the relationships between the kinetics of these three parameters with greater clarity. These figures show a wave of ENE 2A positive alveolar macrophages that appear in BALF 24 h after challenge as neutrophil numbers in the airspaces are declining rapidly. The true significance of the contribution of macrophage phagocytosis to neutrophil clearance cannot be estimated from these data alone because, as demonstrated in Figure 6.2, absolute macrophage numbers were markedly reduced from 5 h until at least 7 days after challenge. However this apparent net egress of macrophages takes no account of possible trafficking of these cells into as well as out of the airspaces. Consequently, if the population of macrophages that had engulfed one or more apoptotic neutrophils rapidly migrated from the airspaces with their newly ingested prey and at least a proportion of them were replaced by an influx of rapidly differentiating monocytes/macrophages, the load of apoptotic neutrophils cleared by this route would be greatly underestimated. In this context, it is interesting to note that there was a significant increase in the proportion of apoptotic neutrophils in BALF at 4 days after challenge (Figure 6.15b), i.e. after the peak in ENE 2A positive macrophages numbers was recorded. As a large wave of apoptotic neutrophils was not observed in parallel with peak numbers of phagocytic macrophages (24 h, Figure 6.15b), this route of neutrophil disposal would appear to be highly efficient.

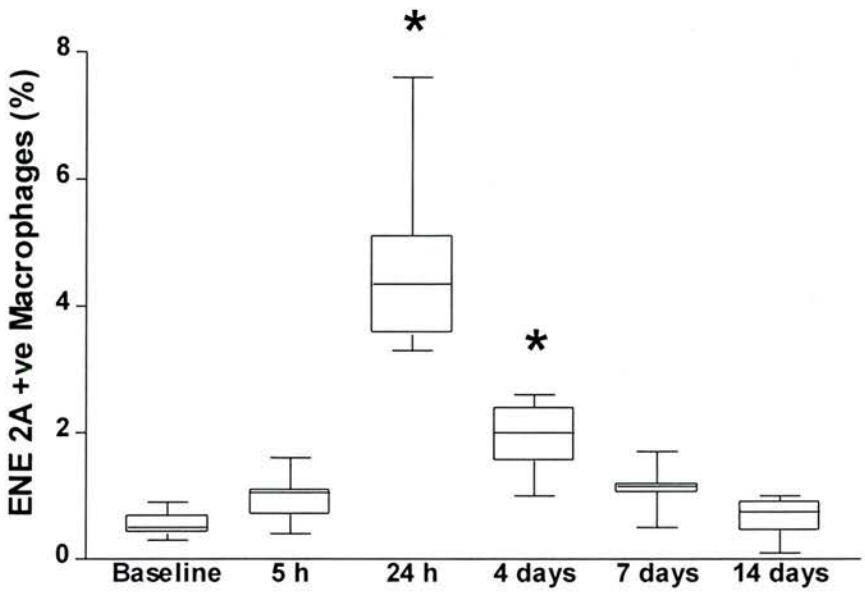

Figure 6.14: Kinetics of macrophage clearance of apoptotic neutrophils *in vivo*
BALF was collected 10 days before (baseline) and at the time points indicated after challenge. Cytocentrifuge preparations were stained immunohistochemically for the presence of ENE 2A. At each time point 500 alveolar macrophages were examined on duplicate slides and the proportion containing ENE 2A positive cell remnants was recorded. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (* ; $p<0.05$ compared to baseline values, n = 6)

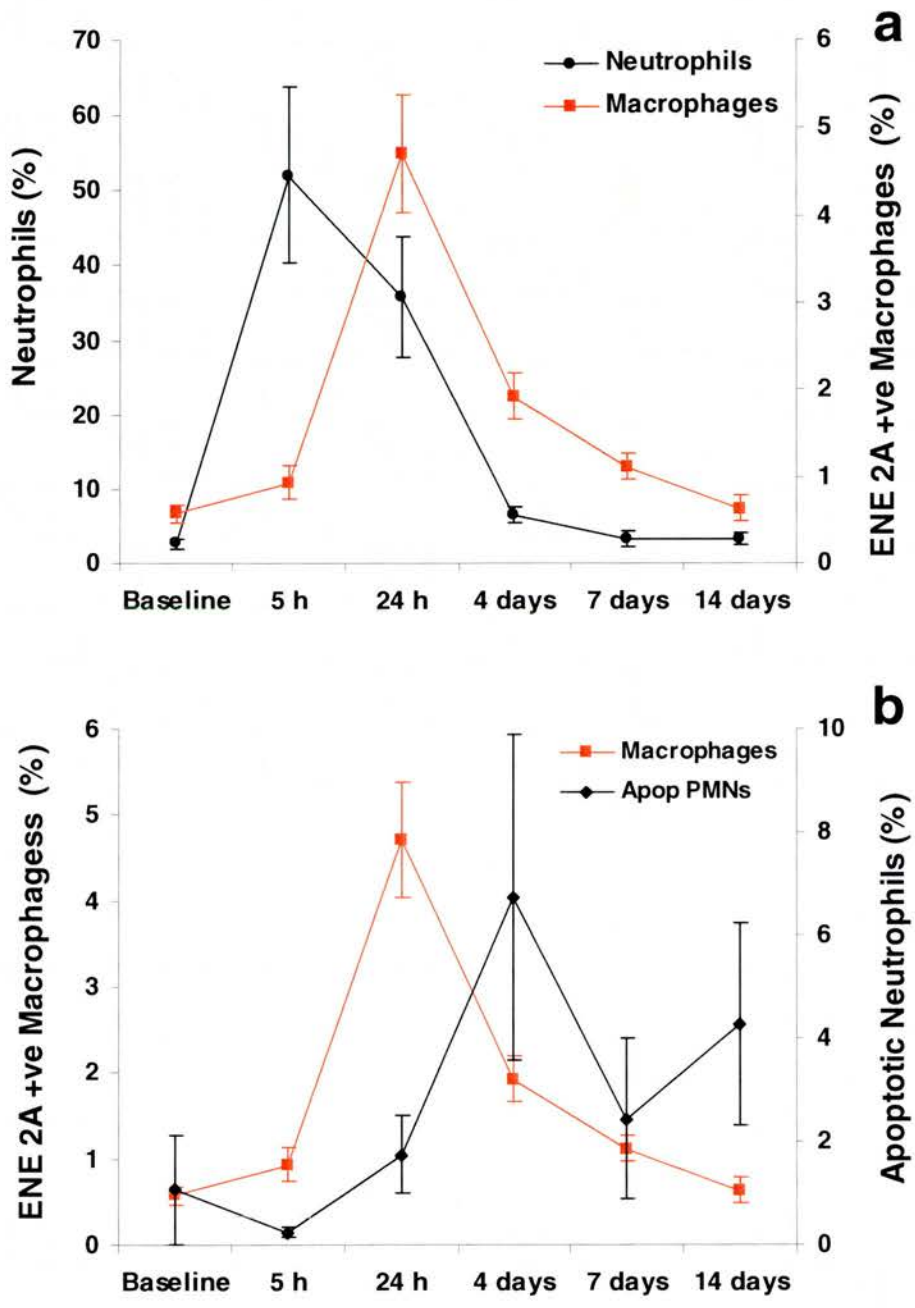

Figure 6.15a,b: Temporal relationships between kinetics of neutrophils, apoptotic neutrophils and ENE 2A positive macrophages in BALF *in vivo*

BALF was collected at the time points indicated. The proportions of neutrophils, apoptotic neutrophils (Apop PMNs) and ENE 2A +ve macrophages were counted on Leishman's, Diff Quik, and immunohistochemically stained cytocentrifuge preparations, respectively. For improved clarity data are presented as mean ± SEM values (n = 6).

a: relationship between differential neutrophil count and proportion of ENE +ve macrophages

b: relationship between proportion of apoptotic neutrophils and ENE +ve macrophages

However, after 4 days, when fewer ENE 2A positive macrophages were present, greater proportions of apoptotic neutrophils were observed (Figure 6.15b) even though their absolute numbers had fallen (Figure 6.11a). This finding could indicate a lower net phagocytic capacity of the population of alveolar macrophages resident in the airspaces at this stage in the resolution of the inflammatory response. Further studies of equine alveolar macrophage phenotype and function will be required to address this hypothesis.

Although the number of macrophages that had ingested neutrophils or their remnants was quantified by the use of a neutrophil specific marker (ENE 2A), cytopathologic features consistent with this process were also observed on Diff Quik-stained slides (Figure 6.10b). To determine how specific these findings in Diff Quik-stained cells were for the presence of ingested neutrophils, a correlation between the two sets of data was examined. The proportion of alveolar macrophages determined to have ingested neutrophil remnants on Diff Quik-stained preparations was significantly correlated with the proportion identified by the specific immunohistochemical method ($r = 0.7$, $p<0.0001$). There was however, a tendency to slightly overestimate the number of phagocytic macrophages on Diff Quik-stained slides suggesting that the densely staining intracytoplasmic chromatin fragments observed may, in some cases, originate from cells other than neutrophils.

6.2.5.4 Effect of BALF supernatant on the rate of constitutive apoptosis in peripheral blood neutrophils *in vitro*

Studies using synovial fluid from human rheumatoid arthritis patients (Bell *et al.*, 1995) and BALF from rabbits with experimental bacterial pneumonia (Lawson *et al.*, 1998b) have demonstrated that during the resolution phase of inflammation a humoral factor is expressed with the capacity to induce apoptosis in peripheral blood neutrophils *in vitro*. The implication that apoptosis of the neutrophil burden is facilitated by a switch in the local cytokine/mediator profile to promote clearance was examined in the current equine COPD model. Peripheral blood neutrophils isolated from healthy normal horses were cultured with BALF supernatants harvested from COPD-susceptible horses at the previously described study time points and with NaCl as a control. Apoptosis was assessed in neutrophils after 8 and

20 h in culture. After 8 h in culture the rate of apoptosis was unaffected by any of the BALF supernatants. After 20 h however, Kruskal-Wallis testing identified a significant difference amongst the groups ($p<0.01$). Comparison of apoptosis in cells cultured with BALF from each time point with control cells (NaCl) demonstrated a significant inhibition of apoptosis at 5 and 24 h after challenge ($p<0.05$, $n = 6$, Figure 6.16). At no time point after challenge did BALF induce apoptosis in peripheral blood neutrophils. The presence of inflammatory mediators, such as IL-8 (Figure 6.9), that might inhibit apoptosis would be expected at 5 h post-challenge when neutrophil numbers were maximal. However the continuing inhibition at 24 h would suggest that clearance of the airspace neutrophil burden and cessation of further neutrophil recruitment occur in spite of the continuing influence of other such pro-inflammatory mediators.

6.3 DISCUSSION

Migration of leucocytes from the pulmonary circulation into normal lung tissue is a dynamic process supplying monocytes/macrophages for clearance of non-self particles and lymphocytes for immune surveillance, but the movement of neutrophils from the vascular space into the pulmonary tissue only occurs in response to an inflammatory stimulus (Hogg and Doerschuk, 1995). Indeed, normal horses have only a small proportion of neutrophils (< 5%) in their BALF (Sweeney *et al.*, 1992a; McGorum and Dixon, 1994; Sweeney *et al.*, 1994; Dixon *et al.*, 1995c; Hobo *et al.*, 1997). Moreover, healthy horses kept permanently at pasture where dust levels are very low may have few if any neutrophils recoverable in BALF (P.M Dixon, B.C. McGorum, R.S. Pirie and T.J. Brazil, unpublished observations). Neutrophils that are observed in the respiratory secretions of horses without any history or clinical signs of lung disease may reflect a particular sensitivity of the horse to non-specific irritants.

Bronchoalveolar lavage is now used routinely in equine clinical practice to sample cell populations in the lower respiratory tract of the horse (McGorum and Dixon, 1994; Moore and Cox, 1996). In developing a protocol for repetitive BAL, the

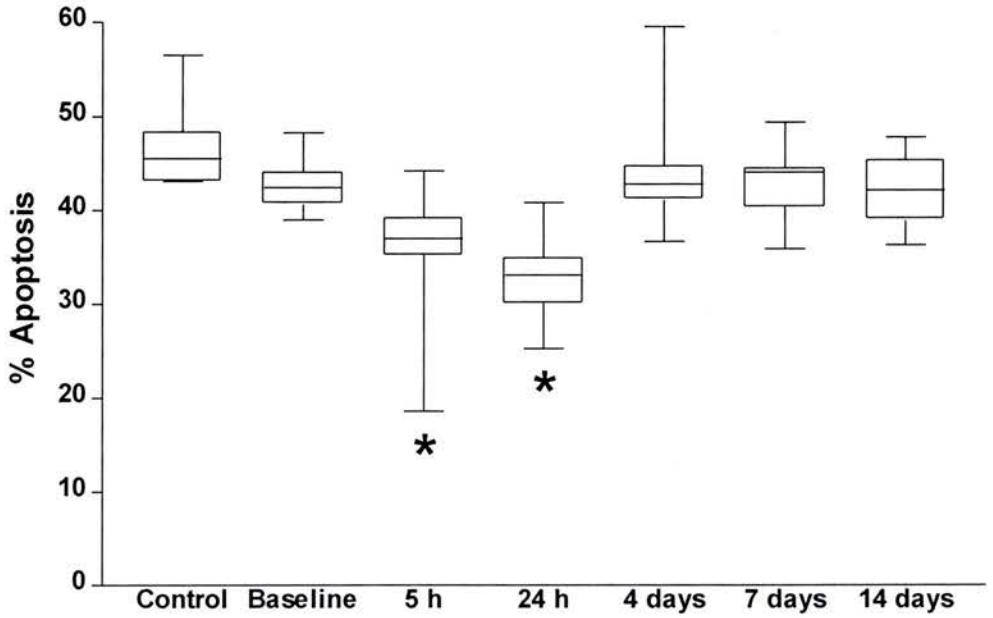

Figure 6.16: Effect of BALF supernatant on apoptosis in peripheral blood neutrophils
Equine peripheral blood neutrophils from healthy horses (5 x 10^6/ml) were incubated in MF at 37°C for 20 h in the presence of NaCl (Control) or BALF supernatant collected 10 days before (Baseline) and at the time points indicated after hay/straw challenge of COPD-susceptible horses. Cells were resuspended and percent apoptosis assessed morphologically on Diff-Quik-stained cytocentrifuge preparations. Data are presented as box and whisker plots indicating median values, 25th and 75th percentiles and maximum and minimum values. (*; $p<0.05$ compared to control values, n = 6)

effects of the previous lavage on the make-up and function of the cell populations harvested at subsequent time points was critically evaluated. In humans (Von Essen *et al.*, 1991) and experimental animal species (Cohen and Batra, 1980; Krombach *et al.*, 1985; Haley *et al.*, 1989; Murray, 1998) a single BAL can induce significant changes in both local pulmonary and systemic neutrophil numbers and function associated with the release of chemotactic factors by resident airway cells (Cohen and Batra, 1980; Teran *et al.*, 1996; Krause *et al.*, 1997). Although studies in humans (Von Essen *et al.*, 1991), dogs and monkeys (Cohen and Batra, 1980) have indicated that a single lavage can induce inflammation diffusely within the lung, in general, the pulmonary neutrophil response is localised to the lung segment lavaged (Krombach *et al.*, 1994) with minor changes occasionally observed in immediately adjacent segments (Weiss *et al.*, 1983). The induced pulmonary neutrophilia resolves over the ensuing 48 h (Pinsker *et al.*, 1980; Weiss *et al.*, 1983; Haley *et al.*, 1989) to 72 h (Von Essen *et al.*, 1991), with the increases in peripheral blood neutrophil numbers and function returning to normal levels within 24 h (Krombach *et al.*, 1985; Von Essen *et al.*, 1991). The localised inflammatory response to BAL is not solely related to saline lavage as bronchoscopy alone has been shown to induce a similar response in both sheep (Weiss *et al.*, 1983) and humans (Krause *et al.*, 1997). Sweeney *et al.* (1994) reported a similarly localised neutrophilic response to BAL in horses, which lasted for at least 48 h but did not affect adjacent airways. However, BALF cell function was not addressed in their study. Several serial BAL protocols were evaluated in the current study. Performing 3 lavages with 0.9% NaCl within 48 h (0 h; right lower segment, 19 h; left lower segment 43 h; right upper segment) did result in more diffuse pulmonary inflammation with increased numbers of neutrophils in the 43 h BAL in 2/3 horses. This is in contrast to a study by Derksen *et al.* (1987), who found no changes in BALF or blood cell counts when performing 3 lavages in different lung segments of normal ponies over a 48 h period. These workers used phosphate buffered saline, which may be less irritant than the more acidic (pH 5.7) 0.9% saline (Sweeney *et al.*, 1994) used in this pilot study. However, the final protocol employed (Section 6.2.1.1) had no significant effect on BALF cell numbers or their CL activity indicating that, with care, serial segmental BAL can be performed in the horse without affecting other lung segments. In calves, a single

lavage can induce changes in alveolar macrophage function (superoxide anion generation, antibody dependent cellular cytotoxicity) in the lavaged segment for between 2 and 6 days (Ohmann and Babiuk, 1986). In the current study, not returning to previous BAL sites for at least 10 days obviated any potential longer term effects on alveolar macrophage function. This was borne out by the sham BAL Luci-DCL studies (Section 6.2.1.1) and the return of absolute alveolar macrophage numbers to baseline levels by 14 days after hay/straw challenge (Figure 6.2). Previous work in this laboratory has shown that in equine COPD the pulmonary inflammatory response is homogenous throughout different lung segments such that a single BAL sample is representative of the entire lung at a particular time point (McGorum *et al.*, 1993e).

To maximise the cell yield during BAL, 0.9% NaCl (without divalent cations) was used to aid cell detachment (particularly of alveolar macrophages) from the airway mucosa and alveolar walls (Brain and Frank, 1973; Naylor *et al.*, 1992). Using lavage fluid at 37°C may help reduce coughing and bronchospasm (Klech and Pohl, 1989; Rennard *et al.*, 1998). Recovery of BALF was approximately 50% of the volume instilled and the volume recovered did not differ significantly after challenge of COPD-susceptible horses as reported previously (Derksen *et al.*, 1985b; Sweeney *et al.*, 1992a; McGorum *et al.*, 1993d; Rush *et al.*, 1998a). BALF cell counts were performed by haemocytometer after removal of mucus and debris by filtration through nylon gauze (which did not affect BALF cell counts, Section 2.8.1). Counts were performed prior to centrifugation to improve accuracy and minimise cell losses (Klech and Pohl, 1989).

An increase in the proportion of neutrophils (> 5%) in BALF concurrent with exposure to hay and straw has become recognized as the cytological hallmark of COPD in susceptible horses in both experimental studies (Derksen *et al.*, 1985b; McGorum *et al.*, 1993d; Rush *et al.*, 1998a) and clinical surveys (Viel, 1983; Winder *et al.*, 1991; Winder *et al.*, 1991; Naylor *et al.*, 1992; McGorum and Dixon, 1994). After hay/straw challenge total BALF cell counts increased due to an influx of neutrophils (Table 6.2). This is consistent with a similar experimental hay/straw challenge study (McGorum *et al.*, 1993d) and one retrospective equine hospital practice study (Vrins *et al.*, 1991). Other experimental studies using much longer

challenge periods (3-7 days) have not observed increased total BALF cell counts in spite of elevated neutrophil counts (Derksen *et al.*, 1985b; Rush *et al.*, 1998a), suggesting that the relative trafficking, and hence proportions of the constituent cell types in BALF may vary depending upon the duration of challenge. Indeed, an initial indication of this is gained from the net decline in the numbers of the other predominant cell types in BALF, macrophages and lymphocytes, at various time points after challenge (Table 6.2).

Apart from the obvious fall in the relative proportions of the other major component BALF cell types (namely lymphocytes and macrophages) following neutrophil influx, absolute numbers of these cells were also reduced significantly after challenge (Table 6.2). Similar reductions in absolute macrophage counts have been reported in previous experimental challenge studies of COPD-susceptible (Derksen *et al.*, 1988; McGorum *et al.*, 1993d; Hare *et al.*, 1999) or artificially sensitised horses (Derksen *et al.*, 1987) and in other lung diseases characterised by pulmonary neutrophilia, such as bacterial pneumonia and pleuropneumonia in the horse (Rossier *et al.*, 1991), bacterial pneumonia in mice (Kooguchi *et al.*, 1998) and ozone exposure in dogs (Fabbri *et al.*, 1984).

This decline in absolute macrophage numbers in BALF is particularly intriguing and interplay among several mechanisms may be involved. Firstly, an increase in the numbers of macrophages that are tightly adherent to the airway walls (Lavnikova *et al.*, 1993) or any obstruction of bronchioles (Robinson *et al.*, 1996) may reduce the numbers of alveolar macrophages that can be aspirated from the distal small airways and alveoli. Secondly, numbers may be depleted by macrophages undergoing apoptosis following challenge and subsequently being phagocytosed by adjacent alveolar macrophages (Khelef *et al.*, 1993; Bingisser *et al.*, 1996; Hussain *et al.*, 1998). Finally, emigration of alveolar macrophages from the airways after the initial inflammatory response to challenge, perhaps to draining lymph nodes (Lan *et al.*, 1993; Bellingan *et al.*, 1996; Hughes *et al.*, 1997) could lead to a decline in macrophage numbers. The study by Bellingan and co-workers (1996) using semi-allogeneic adoptive transfer of inflammatory macrophages, elegantly demonstrated rapid and directed emigration of these cells (within 96 h) to the draining lymph nodes in the resolution phase of experimentally induced peritonitis in mice. Furthermore, a

study of anti-glomerular basement membrane glomerulonephritis (Lan *et al.*, 1993) gives further evidence that significant trafficking of macrophages from an inflammatory focus to local draining lymph nodes may be a general feature of macrophage population dynamics during inflammation. Indeed, although the clearance of radiolabeled neutrophils via lymphatics in the resolution of experimental glomerular capillary injury in rats is minimal, a small neutrophil signal was detected in draining lymph nodes that was exclusively co-localised with macrophages (Hughes *et al.*, 1997). Thus, although the purpose of macrophage emigration is unclear, it may facilitate processing of engulfed material including neutrophils and presentation of antigens to lymphocytes within the lymph nodes (Lan *et al.*, 1993; Bellingan *et al.*, 1996).

The reduction in absolute lymphocyte numbers at 24 h is likely to be a reflection of the considerable traffic of these cells through the airspaces that is known to occur in COPD-susceptible horses during and after antigen challenge (McGorum *et al.*, 1993b; Watson *et al.*, 1997; Rush *et al.*, 1998a). Although increases in lung eosinophil numbers (measured in BALF) (Derksen *et al.*, 1985b) and eosinophil recruitment (monitored by external scintigraphy) (Fairbairn *et al.*, 1993) have been reported in small sub-groups (2/6 and 2/13 horses in these two studies respectively) of COPD-susceptible horses following hay/straw challenge, eosinophil numbers were unaffected by challenge in the current study consistent with previous work in this laboratory (McGorum *et al.*, 1993d).

This is the first study to specifically address the kinetics of air space neutrophil recruitment and clearance following hay/straw challenge of COPD-susceptible horses. By hourly external monitoring of In^{111}-labelled peripheral blood neutrophils, Fairbairn and co-workers (1993b) demonstrated recruitment of neutrophils to the lungs of COPD-susceptible horses within 4 h of the onset of hay/straw challenge. Recruitment reached a plateau by 6 h but monitoring was not pursued beyond 7 h. Increased numbers of neutrophils are evident in BALF by 5 h (McGorum *et al.*, 1993d) and their numbers remain elevated after 3-7 days of continuous hay/straw challenge (Derksen *et al.*, 1987). Although the very early kinetics of neutrophil recruitment to the airspaces following challenge has not been documented in equine COPD, evidence from other models of neutrophilic airway inflammation

demonstrate the rapidity of the neutrophilic response to pulmonary insult. For example, in mice, after inhalation of grain dust, BALF neutrophil numbers increased almost 100 fold to a peak 1 h later and by 4 h had already fallen by 50% (Deetz *et al.*, 1997). Similarly, BALF neutrophil numbers were increased 30 min after intrapulmonary instillation of C5 fragments in rabbits and reached a peak at 4 h (Doherty *et al.*, 1988). However, following direct intratracheal instillation of LPS in mice, BALF neutrophil numbers peaked somewhat later at 6-24 h (Xing *et al.*, 1993; Cox *et al.*, 1995). Perhaps more pertinent to the study of equine COPD which is believed to be associated with a delayed hypersensitivity response (Robinson *et al.*, 1996), the intense pulmonary neutrophilia observed in the ovalbumin sensitised rat model of allergic asthma does not reach its peak until 24 h after aerosol ovalbumin challenge (Schneider *et al.*, 1997). Although direct comparison of varying models of neutrophilic inflammation in different species is unwise, a clear pattern begins to emerge with an initial rapid (within hours) neutrophil recruitment phase after pulmonary insult being followed by a precipitous loss of further chemoattractant activity. Indeed, studies of acute arthritides induced by intra-articular C5 fragments in rabbits (Haslett *et al.*, 1989a) or carageenin (Auer *et al.*, 1990) and LPS (Hawkins *et al.*, 1993; Gottschalk *et al.*, 1998) in horses, demonstrate a rapid cessation of further neutrophil influx within 2-8 h of the initiating insult. In the rabbit model (Haslett *et al.*, 1989a), the rapid fall-off in neutrophil recruitment was due to a time-dependent loss of *in vivo* chemoattractant activity from the synovial cavity. Although in an early study (Derksen *et al.*, 1987), 2/6 COPD-susceptible horses continued to have elevated BALF neutrophil counts 7 days after termination of challenge, no other workers have performed sequential BAL at time points beyond 5 h to monitor the resolution of the pulmonary neutrophilia. The kinetics of the resolving BALF neutrophilia, with a reduction in neutrophil numbers at 24 h and a return to baseline levels by 4 days (Table 6.2) followed a very similar time course to models of neutrophilic pulmonary inflammation induced by grain dust in humans (Deetz *et al.*, 1997) and mice (Wohlford-Lenane *et al.*, 1999), LPS in rodents (Xing *et al.*, 1993; Cox, 1996; Wohlford-Lenane *et al.*, 1999), C5 fragments in rabbits (Doherty *et al.*, 1988), oleic acid in rats (Hussain *et al.*, 1998), ovalbumin in sensitised rats (Schneider *et al.*, 1997) and guinea pigs (Underwood *et al.*, 1995) and

bacillus Calmette-Guerin hypersensitivity in mice (Shellito *et al.*, 1987). These comparative data suggest that the time course of clearance of a population of neutrophils recruited to the airspaces by a single self-limiting insult has a consistent pattern irrespective of the model studied. Indeed, it is likely that the kinetics of such a response is governed, at least in part, by the relatively short lifespan of the neutrophil (see Chapter 4). Once further neutrophil recruitment has ceased, the resident population of neutrophils is cleared from the airspaces.

The pathogenetic impact of airway neutrophil accumulation cannot be extrapolated from their presence in BALF alone and investigation of the functional status of the recruited cells is paramount (Sibille and Reynolds, 1990; Pittet *et al.*, 1997; Jones *et al.*, 1997). As discussed in Section 2.1 and Chapter 3, neutrophils are exquisitely sensitive to functional modulation by inappropriate handling or preparative procedures. To ensure that BALF collection and processing *per se* did not artificially modulate neutrophil function, BALF was collected and processed consistently and rapidly (usually < 60 min) throughout all the challenge experiments. Work from human studies suggests that BALF cell function is stable for up to 4 h (Klech and Pohl, 1989; Rennard *et al.*, 1998). PMA- and fMLP-stimulated superoxide anion generation in human and sheep blood neutrophils has been shown to be unaffected by incubation at room temperature for up to 3 h in the presence of either saline or BALF supernatant from normal human subjects (Nguyen *et al.*, 1989). Furthermore, superoxide anion generation by human peripheral blood neutrophils was unchanged after a 60 min co-incubation with alveolar macrophages from patients with ARDS (Martin *et al.*, 1991). Also, the *in vitro* sham lavage experiments (reported in Sections 2.8.4 and 2.8.5) using peripheral blood neutrophils isolated in a manner to minimise cell priming demonstrated that the trauma of BALF collection itself did not prime neutrophils nor induce degranulation. Thus the *ex vivo* respiratory burst activity of cells recovered from the lower airways as assessed by CL was considered to be a valid indicator of the functional status of the resident cell population *in vivo*.

In the absence of a reliable technique for isolating neutrophils from the mixed cell population in equine BALF, luminigenic probe-dependent CL offered a compromise solution for assessing neutrophil-specific respiratory burst activity (Williams and Cole, 1981; Ward *et al.*, 1990). Despite some detailed studies demonstrating the

myeloperoxidase-dependence of luminol-enhanced CL in neutrophils (Dahlgren and Stendahl, 1983; Allen, 1986) and the identification of neutrophils as the exclusive source of Lum-DCL within mixed populations of human BALF cells (Ward *et al.*, 1990; Williams and Cole, 1981)), a number of early reports also documented significant Lum-DCL activity in adhesion purified populations of alveolar macrophage from both humans (Cluzel *et al.*, 1987; Calhoun and Bush, 1990) and horses (Dyer and Leid, 1983). However, even with greater understanding of the biochemical mechanisms underlying the generation of Lum-DCL, these latter data remain unexplained and continue to stimulate controversy within the literature (Ward *et al.*, 1990; Stevens *et al.*, 1995b). Certainly, stimulus-dependent Lum-DCL measured following addition of purified peripheral blood neutrophils to mixed BALF cells demonstrated a tight correlation between neutrophil numbers and luminol-dependent CL (Humans [Ward *et al.*, 1990]; $r^2 = 0.99$, horses [Section 2.8.3.4, Figure 2.8a,b]; $r^2 = 0.81$). Moreover, studies on a group of human patients with various inflammatory lung diseases demonstrated a similarly tight association ($r = 0.91$) between *ex vivo* Lum-DCL activity and the proportion of neutrophils in mixed BALF cell populations (Williams and Cole, 1981). However, despite their *in vitro* data suggesting that the Lum-DCL response of mixed BALF cell populations reflected only neutrophil activity, Ward and colleagues (1990) found a weaker but still significant correlation between the proportion of neutrophils in BALF from human asthmatics and *ex vivo* Lum-DCL responses than that computed following addition of allogenic peripheral blood neutrophils to normal human BALF. This disparity concurs with the current findings with equine BALF and the levels of correlation between neutrophil numbers and BALF cell Lum-DCL responses in *Ascaris suum*-challenged allergic dogs (Stevens *et al.*, 1995b) and human patients with acute pneumonia (Dalhoff *et al.*, 1994).

The reduced strength of the correlation between BALF neutrophil numbers and Lum-DCL *in vivo* may reflect heterogeneity in the functional status of neutrophils resident in the air spaces at a particular time point (Ward *et al.*, 1990) or could suggest that an alternative source of myeloperoxidase was present in BALF. Eosinophil peroxidase can also generate Lum-DCL (Shult *et al.*, 1986), but the consistently low proportion of eosinophils present in BALF in the current study tends to rule out a confounding

effect from eosinophils. Although no information could be found in the literature regarding the peroxidase content of normal equine alveolar macrophages, none is detectable in human alveolar macrophages (Nichols *et al.*, 1971) including cells harvested from stable asthmatics (Ward *et al.*, 1990). However, during neutrophilic pulmonary inflammation, alveolar macrophages can acquire peroxidase activity *in vivo* by the uptake of soluble myeloperoxidase (Shellito *et al.*, 1987), apoptotic neutrophils (Grigg *et al.*, 1991; Cox *et al.*, 1995) or their remnants (Shellito *et al.*, 1987). Despite being unable to release their acquired myeloperoxidase load (Shellito *et al.*, 1987), alveolar macrophages may utilise this internalised enzyme to enhance killing of micro-organisms that are subsequently phagocytosed (Ramsey *et al.*, 1982). Acquired myeloperoxidase activity may permit alveolar macrophages to contribute to the Lum-DCL generated by a mixed population of BALF cells, thereby reducing the specificity of the assay for neutrophil metabolic activity. Functional studies of purified equine alveolar macrophages before and after challenge are necessary to address this hypothesis.

Notwithstanding the proposed difficulties in the interpretation of equine BALF cell CL activity, several critical points regarding the respiratory burst activity of BALF cells following hay/straw challenge can be made with confidence. Firstly, hay/straw challenge leads to a large increase in the respiratory burst capacity of BALF phagocytes. Secondly, the time course of this enhanced responsiveness is intimately associated with the presence of neutrophils in the airspaces and agonist-stimulated Lum-DCL data suggest that neutrophils are the origin of many of the ROS released. Thirdly, in light of the requirement for priming to induce functional fMLP receptor coupling in equine neutrophils (Section 3.2.3.2), the magnitude of the fMLP-stimulated Lum-DCL responses at 5 and 24 h after challenge would indicate that recruited neutrophils are "heavily" primed *in vivo*. This is further supported by the marked PMA-stimulated Lum-DCL responses at 5 and 24 h (Figure 6.5b) and the enhanced fMLP-stimulated Lum-DCL response of pulmonary neutrophils in comparison to peripheral blood neutrophils (Figure 6.7).

Although several studies have demonstrated changes in the phagocytic activity of BALF phagocytes in horses with chronic pulmonary disease (Nuytten *et al.*, 1983; Klucinski *et al.*, 1994), only a single study investigating their respiratory burst

activity was found in the literature (Olszewski and Laber, 1993). This latter study, using pooled tracheal and BALF cells collected from a heterogeneous population of clinical cases of COPD, found an increase in the spontaneous production of ROS in BALF cells compared to mixed blood leucocytes using the nitroblue tetrazolium method. However, the animals were not studied whilst in remission nor was the BALF phagocyte respiratory burst activity compared to that of cells collected from healthy control animals, making these findings difficult to interpret.

Increases in airspace cell respiratory burst activity are a common feature of pulmonary inflammatory diseases including acute pneumonia (Dalhoff *et al.*, 1994; Jones *et al.*, 1997), ARDS (Ching-Chi and Ching-Yuang, 1992), sarcoidosis (Kelly *et al.*, 1988a) and asthma (Cluzel *et al.*, 1987; Kelly *et al.*, 1988b; Calhoun and Bush, 1990; Ward *et al.*, 1990; Taylor *et al.*, 1996) in humans, allergic airway disease in sensitised dogs (Stevens *et al.*, 1995a; Stevens *et al.*, 1995b) and experimental *Streptococcus pneumoniae*- and bleomycin-induced lung inflammation in rabbits (Jones *et al.*, 1994). However, this is by no means a consistent feature in all inflammatory lung diseases, as BALF neutrophil function may also be significantly compromised in human patients with ARDS (Martin *et al.*, 1991).

Until recently, investigation of the metabolic activity of individual BALF cell types has required physical *ex vivo* isolation of cell populations, for example by adherence purification of alveolar macrophages (Cluzel *et al.*, 1987; Calhoun and Bush, 1990) or use of differential density gradients (Ching-Chi and Ching-Yuang, 1992; Delclaux *et al.*, 1997). In spite of the difficulties described earlier, luminol-dependent CL, as employed in this study has permitted analysis of neutrophil function when physical isolation of neutrophils has been unreliable. Luminol-DCL has proved valuable in the assessment of BALF neutrophil function in *Ascaris suum* sensitised dogs after antigen challenge (Stevens *et al.*, 1995a; Stevens *et al.*, 1995b) and in a number of human lung diseases (Williams and Cole, 1981) including asthma (Kelly *et al.*, 1988b; Ward *et al.*, 1990) as well as in the current equine COPD model. However, all these methods rely heavily on the assumption that the *ex vivo* activity of neutrophils is a true reflection on their behaviour *in situ*. The recognition that within a mixed population of BALF cells, alveolar macrophages may both inhibit neutrophil respiratory burst activity (Sibille and Reynolds, 1990) and scavenge ROS released by

neutrophils (Martin *et al.*, 1991) may further explain the reduced specificity of this technique in some situations.

The functional status of neutrophils within the airspaces is highly dependent on the initiating cause of the lung inflammation, which determines the presence, nature and concentration of neutrophil-activating stimuli within the inflammatory milieu (Ward and Mulligan, 1995; Pittet *et al.*, 1997). The development of the non-metabolisable glucose analogue 2-[^{18}F]-fluoro-2-deoxy-D-glucose as positron emission tomography imaging agent (Taylor *et al.*, 1996) has permitted not only the measurement of neutrophil specific metabolic activity *in situ* (Jones *et al.*, 1994) but has also highlighted the dissociation between emigration of neutrophils into the airways and their subsequent post-migratory respiratory burst activity (Jones *et al.*, 1997). Indeed, this method has demonstrated a link between the persistence of neutrophil metabolic activity in the airways of humans and the development of chronic pulmonary inflammation, scarring and lung fibrosis (Jones *et al.*, 1994; Jones *et al.*, 1998). If such technology could be harnessed for use in the horse, great progress could be made in the investigation of the cellular pathogenesis of both experimentally-induced COPD and the heterogeneous population of animals with longstanding lung inflammation encountered in equine clinical practice, some of which have evidence of irreversible lung injury.

The apparent reduction in the fMLP-stimulated Lum-DCL activity of 1 x 10^6 BALF neutrophils in comparison to 1 x 10^6 blood neutrophils at 24 h and 4 days after challenge (Figure 6.7) raises several interesting questions as to the functional capabilities of airspace neutrophils during this phase of rapid neutrophil clearance. Firstly, the residual neutrophils present in the airways at these time points may have already undergone a sustained period respiratory burst activity and henceforth simply be either desensitised to, or incapable of further stimulation (Pittet *et al.*, 1997). Perhaps of more interest, is the possibility that, at these time points, a significant proportion of the neutrophils present in the airspaces may be undergoing apoptosis. Previous work with human neutrophils (Whyte *et al.*, 1993b) and the data presented in Section 4.2.1.6 and Figure 4.6 have demonstrated that initiation of the apoptotic programme in neutrophils is associated with a reduction in receptor-mediated respiratory burst activity. Indeed, the significant increase in the absolute numbers of

free apoptotic neutrophils and the proportion of ENE 2A positive alveolar macrophages at 24 h and 4 days would support this hypothesis. However, further studies of isolated BALF neutrophils collected at these time points after challenge would be necessary to address this hypothesis in detail.

Although elevated levels of proteolytic activity have been previously demonstrated in the tracheal secretions of horses with COPD (Koivunen *et al.*, 1996; Koivunen *et al.*, 1997a; Koivunen *et al.*, 1997b; Raulo and Maisi, 1998), the increased concentration of ENE 2A measured in BALF at 5 h, 24 h and 4 days after challenge in the current study (Figure 6.8) is the first recognition of significant neutrophil degranulation and elastase release in the distal airways of horses with COPD. This strengthens the case for neutrophils, and neutrophil elastase in particular, playing a central role in the observed lung injury. Despite these data, previous studies of equine tracheal secretions have suggested that serine proteinases, such as elastase, are unlikely to have significant importance in the pathogenesis of equine COPD (Koivunen *et al.*, 1996). Indeed, the absence of functional elastase activity in BALF (Section 6.2.3), suggesting that the released elastase had been rapidly inactivated *in situ* by serine proteinase inhibitors, such as α-1-proteinase inhibitor (Potempa *et al.*, 1991; Milne *et al.*, 1994), would support this contention. However, the absence of functional elastolytic activity in BALF does not in itself refute a role for neutrophil elastase in lung injury. Although the presence of functional elastase has been reported in the BALF of human patients with acute lung injury (Lee *et al.*, 1981), others have failed to identify such activity (Dent *et al.*, 1995), even in severe pulmonary diseases associated with significant structural damage such as ARDS (Lee *et al.*, 1981; Pittet *et al.*, 1997). In view of the high concentrations of serine proteinase inhibitors in the plasma and the PELF during pulmonary inflammation (Milne *et al.*, 1994; Owen and Campbell, 1999), and the rapid association of these inhibitors with their target proteinases (Owen and Campbell, 1999), it is not at all surprising that I was unable to measure elastase activity in the current study.

In 1989, Weiss suggested that the interaction between antiproteases, ROS and proteases, compartmentalised within the immediate pericellular environment adjacent to a neutrophil and its target, would dictate the destructive capability of neutrophil elastase (reviewed in Section 1.6). In humans, ROS may oxidise and

inactivate α-1-proteinase inhibitor by oxidation of a critical active site methionine residue thereby permitting progression of unbridled, yet highly localised elastase activity (Weiss, 1989; Dallegri and Ottonello, 1997). Although the horse possesses several oxidation-resistant forms of α-1-proteinase inhibitor, other proteases such as MMP-9 (high levels of which were detected in BALF in the current study; P. Maisi and T.J. Brazil, unpublished observations) may also inactivate inhibitors (Owen and Campbell, 1999). Work in human neutrophils has identified several other mechanisms to enable localised pericellular neutrophil elastase activity to persist even in the presence of high affinity proteinase inhibitors (Owen and Campbell, 1999). High local concentrations of proteases may be compartmentalised by tight adhesion between neutrophil or elastase molecules and a target cell or substrate with physical exclusion of the much larger inhibitor molecules (Owen and Campbell, 1999). Such steric hindrance may also underlie the continuing functional integrity of elastase molecules that remain tightly bound to the neutrophil plasma membrane (Owen *et al.*, 1995). Finally, very high levels of proteinases may overwhelm the antiproteinase screen either within a dense focus of neutrophils (Dent *et al.*, 1995; Owen and Campbell, 1999) or in the immediate vicinity of release of an individual elastase-bearing azurophil granule, so-called "quantum proteolysis" (Liou and Campbell, 1996).

Therefore, the presence of significant amounts of elastase in BALF may be of critical pathogenetic relevance. Although significant proteolytic lung damage is rare in equine COPD, the ability of neutrophil elastase to stimulate goblet cell degranulation and mucus hypersecretion in other species (Agusti *et al.*, 1998) might indicate just one potential role for neutrophil elastase in the genesis of airway obstruction in equine COPD (Robinson *et al.*, 1996). Of particular note is the recent demonstration that neutrophil elastase stimulates a time-dependent increase in mRNA expression of the major human respiratory mucin gene, *MUC5AC* in human bronchial epithelial cells (Voynow *et al.*, 1999). Indeed, it was a cross-reacting antibody to human MUC5AC protein, which was used by Jeffcoat *et al.* (1998) and in our own work (A.J. Kemmish, T.J. Brazil and S.D. Carrington, unpublished observations) to demonstrate increased mucin levels in the airways of horses with experimentally-induced COPD.

The current study is also the first to document increased concentrations of the potent neutrophil chemoattractant cytokine, IL-8 (Baggiolini and Clark-Lewis, 1992; Baggiolini *et al.*, 1994) in the BALF of COPD-susceptible horses following hay/straw challenge (Section 6.2.4 and Figure 6.9). The increase in IL-8 at 5 h is consistent with IL-8 production as a contributing signal for local neutrophil accumulation (Baggiolini *et al.*, 1994). Moreover, this finding supports the hypothesis based on *in vitro* data, that proposed a switch in alveolar macrophage function from a primarily phagocytic role to an enhanced secretory role, producing increased levels of the neutrophil chemotactic cytokines IL-8 and MIP-2 in the pathogenesis of the pulmonary neutrophilia observed in equine COPD (Franchini *et al.*, 1998). Although the initial kinetics of IL-8 production closely matched those of the pulmonary neutrophilia, with a peak at 5 h and a decline thereafter (Figure 6.9), further work is necessary to answer several fundamental questions regarding these data. Firstly, the differential between baseline concentrations of IL-8 (when BALF neutrophil numbers were minimal) and those measured at 5 h (peak BALF neutrophil response), although significant, was surprisingly small. Secondly, the concentrations of IL-8 detected in BALF at baseline in the current study (145.2 ng/ml, 61.9-615.4 ng/ml; median and range), despite seeming quite high, are similar to those detected in BALF from both normal and other COPD-susceptible horses whilst in remission (Dr M. Francini, personal communication). However, the concentrations of IL-8 protein in BALF were substantially (50-fold) higher than those measured by specific ELISA in the BALF of high-risk human patients who progressed to develop ARDS (Donnelly *et al.*, 1993). The apparently biphasic release of IL-8 detected in equine BALF is not without precedent with messenger RNA expression in isolated bovine alveolar macrophages showing a biphasic pattern following LPS stimulation, with peaks at 1-2 and 16-24 h (Lafleur *et al.*, 1998). Furthermore, in other species, neutrophils themselves are a significant source of IL-8 that may serve to amplify the inflammatory response by recruiting more neutrophils (Baggiolini *et al.*, 1994). Further detailed investigation of IL-8 production, kinetics and biological activity in the horse is required to address these apparent discrepancies.

Physical mechanisms such as mucociliary clearance and coughing undoubtedly provide an important route for expulsion of at least part of the neutrophil load within

the inflamed lungs (Boggs, 1967; Nicod, 1999). External monitoring of radiolabeled neutrophils during the investigation of lung inflammation has often shown evidence of neutrophil movement up the conducting airways, which are subsequently swallowed and observed within the stomach (Haslett *et al.*, 1989c; Jones *et al.*, 1997). Furthermore, in the current study, tracheal secretions collected immediately after challenge (5 h) were greatly increased in volume and contained massive numbers of neutrophils (T.J. Brazil, unpublished observations).

However, over the last 10 years an ever increasing body of literature has accumulated to provide compelling evidence that apoptosis of neutrophils with subsequent clearance by both macrophages and other "semi-professional" phagocytes also plays a crucial and perhaps primary role in the resolution of neutrophilic inflammation *in vivo* (Haslett, 1997; Savill, 1997a; Savill, 1997b). It would now appear that this pathway from apoptosis of neutrophils, through recognition and engulfment of intact cells by macrophages to complete degradation of the neutrophil's component parts, may represent the normal and physiological ("intended") fate of neutrophils reaching the end of their functional lifespan at sites of inflammation. Light and electron microscopic evidence of this process has been reported in inflammatory diseases of the kidney (Savill *et al.*, 1992), joints (Spiggs *et al.*, 1978; Savill *et al.*, 1989b), gut (Whyte *et al.*, 1997), peritoneal cavity (Sanui *et al.*, 1982) and lungs (Grigg *et al.*, 1991; Cox *et al.*, 1995; Matute-Bello *et al.*, 1997; Lawson *et al.*, 1998a; Pryjma *et al.*, 1999) in man and experimental animals. Also, the onset of neutrophil apoptosis in oleic acid-induced lung injury in rats correlates with general histological evidence of its resolution (Hussain *et al.*, 1998). This author has recognized both free apoptotic neutrophils, and resident macrophages that appeared to have phagocytosed apoptotic neutrophils, in cytological specimens from a variety of naturally occurring, septic and non-septic, inflammatory disorders of the joints, peritoneal cavity, uterus and lungs of the horse (T.J. Brazil, unpublished observations).

In the current study, detection of small numbers of apoptotic neutrophils and ENE 2A positive alveolar macrophages (mean 0.2%) in BALF collected whilst the horses were in clinical remission, suggests that this may be an ongoing physiological process to scavenge effete neutrophils, even during periods of relative pulmonary

health. Small numbers of alveolar macrophages (mean 0.27%) containing apoptotic neutrophils were also found by Cox *et al.* (1995) in normal Sprague-Dawley rats and in BALF (0.6%) from normal rabbits (Lawson *et al.*, 1998a).

The morphological evidence presented in Section 6.2.5 clearly demonstrated that apoptosis and macrophage phagocytosis of neutrophils is a prominent feature in the resolution of the inflammatory process observed in equine COPD. The light and electron microscopic features were very similar to those observed in human neonatal respiratory distress syndrome (Grigg *et al.*, 1991) and ARDS (Matute-Bello *et al.*, 1997) and in lung injury induced experimentally with intratracheal LPS (Cox *et al.*, 1995) or intravenous oleic acid (Hussain *et al.*, 1998) in rats. Also the morphology of non-ingested apoptotic equine neutrophils observed *in vivo* appeared identical to that of neutrophils aged *in vitro* (Section 4.2.1.1).

As discussed earlier, the kinetics of BALF neutrophil recruitment and clearance reported herein were similar to those reported for LPS- (Cox *et al.*, 1995) and oleic acid-induced (Hussain *et al.*, 1998) lung injury in rats and bacterial pneumonia in rabbits (Lawson *et al.*, 1998a); models in which the contribution of neutrophil apoptosis and macrophage phagocytosis to the resolution of pulmonary neutrophilia have been investigated in some detail. However, some differences are noteworthy in the context of the current kinetic data. In the equine COPD model, BALF neutrophil numbers were greatest at 5 h (the end the challenge period), whereas in the rabbit pneumonia model (Lawson *et al.*, 1998a) and following intratracheal administration of LPS or intravenous administration of oleic acid in the rat, neutrophil numbers were maximal at 12, 18 and 24 h, respectively. In all four models however, a wave of apoptotic neutrophils began to appear 4-12 h after the onset of the inflammatory response and peaked at 24 h. In the oleic acid model, apoptotic neutrophils detected by TUNEL constituted nearly 40% of total BALF cells and almost 60% of the neutrophil burden at 24 h (Underwood *et al.*, 1995). In the LPS model, where apoptotic neutrophils were identified by typical morphologic features on Diff Quik-stained slides, these cells accounted for only 1.8% of total neutrophil numbers at 24 h (Cox *et al.*, 1995), a similar proportion to that found in equine BALF at 24 h (Figure 6.11b). Numbers of apoptotic neutrophils in the pneumonia model were intermediate (7.3%). The large numbers of apoptotic neutrophils detected by TUNEL probably

reflects the possibly higher sensitivity and much lower specificity of this technique in comparison to simple light microscopic assessment (Labat-Moleur *et al.*, 1998). These data suggest that neutrophils that undergo apoptosis *in vivo* are most likely recognized very rapidly after the onset of apoptosis, and perhaps more critically, that to prevent leakage of their noxious contents in the post-apoptotic phase, a large neutrophil burden can be recognized and cleared extremely efficiently by local macrophages. This hypothesis assumes that few neutrophils proceed from apoptosis to secondary necrosis whereupon they are no longer identifiable. However, the lack of cytologically detectable cell debris after hay/straw challenge and the very high BALF cell viability as assessed by trypan blue exclusion, would suggest that necrosis of neutrophils *in situ* is a very rare event in equine COPD.

The apparently abrupt switch from recruitment and activation of neutrophils to apoptosis and clearance raised the question as to whether this simply reflected neutrophils reaching the end of their useful life in the airways or was an event orchestrated by some fundamental change in the local mediator profile. The latter explanation has been hinted at by studies with the anti-inflammatory cytokine IL-10. Interleukin-10 attenuates the anti-apoptotic (survival) effect of LPS in human neutrophils *in vitro* (Cox, 1996; Keel *et al.*, 1997) and pre-treatment of mice with IL-10 significantly reduced the neutrophilic response to intratracheal LPS at time points beyond 18 h, suggesting that it facilitated neutrophil clearance (Cox, 1996). In light of the important inhibitory role of some cytokines produced by alveolar macrophages in the regulation of neutrophil apoptosis (Herlihy *et al.*, 1996; Matute-Bello *et al.*, 1997), a recent report of a potent inhibitory effect of hrIL-10 on the production of pro-inflammatory cytokines by LPS-stimulated equine peritoneal macrophages (Hawkins *et al.*, 1998) offers an exciting new avenue of investigation in equine neutrophil biology.

Further evidence for directed neutrophil apoptosis *in vivo* has come from reports of pro-apoptotic activity in human synovial fluid (Bell *et al.*, 1995) and pneumonic rabbit BALF (Lawson *et al.*, 1998b) collected during the resolution phase of an acute inflammatory response. Although, the *anti*-apoptotic effect of BALF fluid collected from horses during the rapid phase of neutrophil clearance would tend to rule out the presence of dominant anti-inflammatory factors, their expression may be short lived.

Collection of BALF at time points between 5 and 24 h after hay/straw challenge of horses would help to address this question.

The use of a highly specific immunological marker for equine neutrophils (ENE 2A) permitted accurate and sensitive quantitation of macrophages containing neutrophil remnants at each timepoint. ENE 2A immunostaining also complemented observations from Diff Quik-stained preparations suggesting that degradation ensued rapidly following phagocytosis of intact cells. Cox *et al.* (1995) reported that histochemical staining of alveolar macrophages for peroxidase activity was almost seven times more sensitive for the detection of intracytoplasmic neutrophil remnants than examination of Diff Quik-stained specimens. However, in that study many peroxidase positive cells had only very diffuse staining, precluding any assertion that this originated from degradation of cells ingested intact. In the current study, alveolar macrophages were only scored as positive if ENE 2A staining was associated with condensed nuclear material or formed tight clusters consistent with engulfment of an entire cell.

Data from interactions between monocyte-derived macrophages and aged neutrophils suggest that recognition and phagocytosis of the apoptotic cells is a rapid event, occurring within minutes of their co-incubation (Newman *et al.*, 1982), with ingested neutrophils being no longer recognizable as such by 1 h (Haslett, 1992). Studies of apoptosis in other cell types confirm the rapidity with which they are eliminated; for example, rat hepatocytes become histologically undetectable within 3 h (range 139-226 min) of induction of apoptosis *in vivo* (Bursch *et al.*, 1990). Although formulae are available for the calculation of rates of cell loss by apoptosis in solid organs such as the liver (Bursch *et al.*, 1990), they should be applied with caution to the dynamic environment of the airways. Without taking macrophage trafficking into account, with such rapid degradation of cells even a very low point prevalence of pulmonary neutrophil apoptosis (either free or newly ingested by macrophages) may equate to a very large capacity for neutrophil elimination.

The kinetics of ENE 2A positive macrophages and their close temporal association with neutrophil disappearance from the airspaces was almost identical to the models described earlier (Cox *et al.*, 1995; Underwood *et al.*, 1995; Lawson *et al.*, 1998a) and to an early study of "neutrophil-phagocytosing macrophages" in a guinea pig

model of self limiting peritonitis (Sanui *et al.*, 1982). This early study by Sanui and colleagues (1982) also suggested that macrophage clearance might have an ongoing role in more chronic inflammation. A second pulse of neutrophils induced by re-injection of peritoneal irritant was followed by a second wave of phagocytosing macrophages that cleared the neutrophil influx over a similar time course.

These data offer a persuasive case for macrophage clearance of redundant neutrophils (without exacerbating the inflammatory response (Meagher *et al.*, 1992), as an almost obligatory step in the successful resolution of an acute inflammatory response irrespective of stimulus or anatomical site (Savill, 1997a). Moreover, a similar paradigm is recognized in the resolution of eosinophilic airway inflammation in experimentally sensitised mice (Kodama *et al.*, 1998) and human asthma (Woolley *et al.*, 1996).

Although the current study has focussed on the regulation of the potentially deleterious effects of the neutrophil, much evidence suggests that in fact, the macrophage may be the critical control point in the successful resolution of the inflammatory process. It is well recognized that increased numbers of alveolar macrophages is associated with increased survival in humans with acute lung injury (Pittet *et al.*, 1997). Blocking macrophage phagocytosis *in vivo* in the guinea pig peritonitis model greatly prolonged local neutrophilia (Sanui *et al.*, 1982). Even more persuasively, in experimental murine pneumonia, depletion of alveolar macrophages *in vivo*, despite slowing neutrophil recruitment, also causes a more prolonged neutrophil response, with reduced bacterial clearance, exaggerated lung injury and increased mortality (Kooguchi *et al.*, 1998). A recent study of children prone to recurrent respiratory infections reported less efficient phagocytosis of apoptotic neutrophils by their alveolar macrophages *in vitro*, both in the presence and absence of active infection in comparison to both normal and asthmatic children (Pryjma *et al.*, 1999).

The seminal studies by Newman *et al.* (1982) demonstrating that the "age" of a neutrophil was critical for it to be recognized by macrophages, gave an initial indication that macrophage phenotype was also critical in this mechanism. They showed that monocyte-derived macrophages and inflammatory lung macrophages readily phagocytosed senescent neutrophils but resident alveolar macrophages did so

only very poorly. More recent *in vitro* studies have demonstrated that maturation of monocytes into macrophages with the capacity for neutrophil clearance, represents a distinct macrophage differentiation pathway (Henson and Riches, 1994). Also the very signals that promote neutrophil recruitment and activation and retard their progression to apoptosis such as GM-CSF and IL-1β also potentiate macrophage phagocytosis of apoptotic neutrophils (Ren and Savill, 1995). A further indication that resolution of the inflammatory response encompasses a complex network of interactive negative feedback loops, comes from data showing that in contrast to other particles, the engulfment of apoptotic neutrophils not only fails to elicit release of pro-inflammatory enzymes, cytokines and thromboxanes (Meagher *et al.*, 1992; Savill, 1997a; Liu *et al.*, 1999) but also actively downregulates further production of pro-inflammatory cytokines such as TNF-α and IL-8 by macrophages (Fadok *et al.*, 1998).

In conclusion, these data provide the first *in vivo* evidence of a pivotal role for neutrophil priming, recruitment and activation with subsequent apoptosis and macrophage clearance in the genesis and resolution of the lung inflammation and injury observed in equine COPD.

CHAPTER 7

SUMMARY AND CONCLUSIONS

It is clear from studies in human neutrophils that to accurately model neutrophil function *in vitro* requires careful preparation and handling of cells throughout the experimental protocol. It was indeed serendipitous that a neutrophil isolation method capable of producing populations of human neutrophils with high purity and minimal cell priming proved to be similarly valuable for the isolation of equine neutrophils. Great care was taken to collect and prepare equine neutrophils in such a way to avoid cell trauma or contamination with even trace amounts of LPS that might cause neutrophil priming. Freshly isolated equine neutrophils prepared in this way showed minimal cell shape change. Although subsequent experiments suggested that, at least *in vitro*, functional priming was not invariably associated with shape change (e.g. LPS), the lack of shape change in freshly isolated equine neutrophils lent support to the choice of isolation method. Assessment of basal (unstimulated) CL activity in freshly isolated neutrophils further suggested that cell function was minimally perturbed during purification. The discovery that in equine neutrophils, priming appeared to be a prerequisite for functional coupling of fMLP receptors, permitted the fMLP-stimulated Luci-DCL response of freshly isolated cells to be used as a gold standard to confirm the absence of priming during cell preparation.

The fMLP-stimulated Luci-DCL response of equine neutrophils was also invaluable in the characterization of their priming by inflammatory mediators (such as LPS, PAF and TNF-α). Recognition that exposure of equine neutrophils to a variety of pro-inflammatory mediators greatly enhances their subsequent secretagogue-induced respiratory burst activity and is likely to have profound effects on their sequestration in the microvasculature *in vivo*, has great implications for the future investigation of equine inflammatory disease pathogenesis.

Circulating lipopolysaccharide or endotoxin is perceived to be central to the pathogenesis of many life-threatening equine diseases, particularly gastro-intestinal obstruction and/or mucosal inflammation, pulmonary infection and acute

endometritis; all diseases with high mortality even in the face of aggressive antibacterial, anti-inflammatory and supportive therapy. It is important to note that in comparison to other mammals, the horse appears to be exquisitely sensitive to the biological effects of LPS *in vivo*. The lethal intraperitoneal dose of LPS in the horse (on a mg/kg basis) is some 20-fold lower than in laboratory animals such as rats, mice, guinea pigs and rabbits (Berczi *et al.*, 1966; Burrows, 1981). The complimentary role of plasma lipolysaccharide binding proteins and CD14 in the recognition of LPS by cells leading to transmembrane signaling is now thought to be central to the pathogenesis of endotoxaemia and septic shock. The enhanced sensitivity of equine neutrophils to LPS priming in the presence of serum (either equine or human) demonstrated in the current study suggests that a similar LBP/CD14 paradigm may be equally important in the horse. Further investigation of the cellular pathophysiology of LPS exposure should be an urgent imperative in equine cell biology research.

The current study provides the first detailed evidence that equine neutrophils undergo constitutive apoptosis when aged in culture. Ageing equine neutrophils developed the archetypal features of nuclear condensation, chromatin fragmentation and plasma membrane phospholipid redistribution recognized in many apoptotic cell types including human neutrophils. Furthermore, the constitutive rate of apoptosis in equine neutrophils can be profoundly altered by exposure to some of the pro-inflammatory mediators that are likely to be active during inflammation *in vivo* and which I had previously characterized as priming or activating agents of equine neutrophil function *in vitro*. The marked inhibition of constitutive apoptosis mediated by glucocorticosteroids in both human (Meagher *et al.*, 1996) and equine neutrophils indicated the capacity that pharmacological intervention may have for modulation of neutrophil longevity and hence function *in vivo*. Indeed, the contrasting, *pro*-apoptotic effect of glucocorticosteroids on human eosinophils *in vitro* has been reported to be, at least in part, responsible for the resolution of airway inflammation in human asthmatics treated with corticosteroids (Woolley *et al.*, 1996). The apparent discrepancy, in terms of both the time- and concentration-dependence of the anti-apoptotic effect of dexamethasone, between the two species' neutrophils remains unexplained but is worthy of further study.

Two other findings from these *in vitro* studies of apoptosis in equine neutrophils are worthy of particular note. Firstly, the pro-apoptotic effect of LPS was entirely unexpected. This effect was shown to be concentration-dependent and independent of autocrine TNF-α activity. Although preliminary characterization suggested that this was a novel species variation, further work will be necessary to confirm these data. One can speculate upon the evolutionary advantage of such a pro-apoptotic effect in a species so profoundly sensitive to the biological effects of endotoxin. Perhaps the induction of apoptosis in equine neutrophils following interaction with LPS prevents additional neutrophil-mediated host tissue damage. The often severe neutropaenia associated with acute equine endotoxaemia *in vivo* (Moore and Barton, 1999) may in part be mediated by early removal of neutrophils in much the same way as has been suggested for the induction of eosinopaenia following corticosteroid therapy *in vivo* (Meagher *et al.*, 1996). Such a response undoubtedly has consequences for efficient antimicrobial defence as evidenced by the propensity for persistently clinically endotoxaemic horses to develop secondary sepsis associated with jugular thrombophlebitis and surgical incisions. As stated above, these contentions remain speculative and await further study of both the complex pathophysiology of equine endotoxaemia and the effects of LPS on equine neutrophils.

The second interesting finding was the marked pro-apoptotic stimulus provided by phagocytosis of immunoglobulin-coated ovine erythrocytes. The obvious question arising from this data is "what would be the effect of phagocytosis of pathogenic bacteria?" Certainly phagocytosis of opsonised *E. coli* provides a similar pro-apoptotic signal for human neutrophils (Watson *et al.*, 1996b). The effect that phagocytosis of the causative agents of important equine bacterial infections (such as *Streptococcus equi*) may have on equine neutrophil apoptosis might provide useful information in the investigation of bacterial pathogenicity.

The *in vitro* studies discussed above serve to initiate understanding of the potential for fine tuning of neutrophil function by cell priming, activation and apoptosis in maintenance of the delicate balance between appropriate and efficient microbicidal activity and host tissue injury. The experimental techniques developed during the

first phase of the project helped to provide answers to several of the fundamental questions regarding neutrophil function *in vivo* in equine COPD as initially proposed in Section 1.8.

Serial study of the fMLP-stimulated Luci-DCL response of peripheral blood neutrophils suggested that these cells become primed in the circulation, in response to hay/straw challenge of COPD-susceptible horses. Simultaneous reduction in the rate of constitutive apoptosis of peripheral blood neutrophils supports *in vitro* studies linking neutrophil priming and longevity (Lee *et al.*, 1993). Whether these effects are due to release of neutrophil priming agents into the circulation or a consequence of cells trafficking through inflamed/activated pulmonary microvasculature or a combination of these potential sources of pro-inflammatory mediators, remains unknown. It was not possible to assess either the priming capability or effects on neutrophil apoptosis of serum collected during the genesis and resolution of the induced disease, but such experiments would yield valuable data.

The current hay/straw challenge studies confirm that neutrophil recruitment to the airways is the key cytological feature of the pulmonary inflammation in equine COPD. Concurrent serial assessment of BALF cell CL demonstrated for the first time that the respiratory burst activity of cells resident in the airspaces was markedly enhanced following hay/straw challenge and agonist-stimulated Lum-DCL responses suggested that recruited neutrophils were responsible for much of this enhanced oxidative activity. Notwithstanding the difficulties inherent to the interpretation of agonist-stimulated Lum-DCL data in mixed BALF cell populations *in vivo* that were discussed in Chapter 6, these data suggest that neutrophils recruited to the airspaces are functionally upregulated.

The BALF cell CL data clearly demonstrates the difficulty in drawing a clear line between priming and activation of neutrophils *in vivo*. The markedly increased basal Lum-DCL of BALF cells harvested from 3/6 horses at 5 h suggests that neutrophils were activated in the airspaces *in vivo*. The detection of large amounts of neutrophil elastase in BALF supernatant after challenge would support this. However, since BALF cells were capable of generating significant agonist-stimulated CL *ex vivo*, it would be reasonable to conclude that at least some of the neutrophils or indeed some of their oxidant generating pathways remained in a primed state with the capacity to

produce an enhanced response upon subsequent secretagogue activation. These arguments are difficult to conclude but may be rationalised by hypothesising that the airspace neutrophil burden is composed of sub-populations of cells with a spectrum of priming/activation states. This hypothesis could be tested by cell-by-cell analysis of basal and agonist-stimulated respiratory burst activity of purified BALF neutrophils in a flow cytometry assay (Chollet-Martin *et al.*, 1992). This, of course, presumes that methods can be developed to purify equine BALF neutrophils without altering their functional status. However, it is clear that at least some neutrophils undergo a respiratory burst and degranulate within the airspaces and this evidence only strengthens the case for neutrophils and their products being central to the pathogenesis of the lung injury observed in equine COPD.

As ever in equine research, the significance of these functional data could be strengthened considerably by the inclusion of more animals in the study population. Moreover, heterogeneity amongst the responses of different COPD-susceptible horses to hay/straw challenge could be more clearly recognized. A particular disappointment in this study was that, due to financial constraints, it was impossible to include a group of healthy control animals. Previous studies from this laboratory have reported that a similar 5 h hay/straw challenge protocol did not induce lung dysfunction or BALF neutrophilia in healthy control animals (McGorum *et al.*, 1993d). However, comparison of the effects of hay/straw challenge on both BALF cell and in particular, peripheral blood neutrophil CL activity with a control population would have greatly increased the strength of the current data, wherein conclusions were reliant upon comparison with cell function whilst COPD-susceptible animals were in remission.

A significant feature of the BALF cell kinetics after hay/straw challenge was the rapidity with which the pulmonary neutrophil burden was cleared after the 5 h challenge period. Although the relative importance of potential neutrophil clearance routes cannot be derived from the current data, this study does substantiate a significant role for neutrophil apoptosis and macrophage clearance in the resolution of equine pulmonary inflammation *in vivo*. Light microscopic, immunohistochemical and ultrastructural examination of BALF cells confirmed that alveolar macrophages ingested apoptotic neutrophils *in vivo*. The appearance of

increased numbers of ENE 2A positive alveolar macrophages as BALF neutrophil numbers declined was striking, in spite of the net reduction in BALF alveolar macrophage numbers for at least 7 days after challenge. The eventual fate of the ingested and degraded neutrophil components is unclear. Although the precise nature of macrophage trafficking through the airways is as yet unknown, it is tempting to speculate that having ingested effete neutrophils, alveolar macrophages migrate from the airways, possibly to draining lymph nodes as suggested by experimental models of peritonitis and glomerulonephritis in rodents (Lan *et al.*, 1993; Bellingan *et al.*, 1996).

Corticosteroid therapy significantly attenuates both airway dysfunction and inflammation (as evidenced by a reduction in BALF neutrophil counts) in symptomatic COPD horses (Rush *et al.*, 1998a; Rush *et al.*, 1998b). In light of the promotion of phagocytosis of apoptotic neutrophils afforded by exposure of macrophages to glucocorticoids *in vitro* (Liu *et al.*, 1999), it would be fascinating to monitor BALF neutrophil apoptosis and macrophage clearance following corticosteroid therapy of COPD-affected horses.

During this study it has become clear that alveolar macrophages may in fact be pivotal to the initiation, amplification and not least the resolution of the pulmonary inflammatory response in equine COPD. Already some progress has been made in defining the cytokine profile of activated equine alveolar macrophages (Franchini *et al.*, 1998) and as recently developed primers for quantitation of equine cytokine mRNA (Giguere and Prescott, 1999) become more widely available, the undoubtedly complex array of cytokines involved in equine inflammation will begin to be unravelled. In the mean time, investigation of equine alveolar macrophage recognition of apoptotic neutrophils *in vitro* would be a valuable endeavour.

Although the results of this study have served to increase understanding of neutrophil function in equine COPD, assessment of the precise role of the neutrophil in the pathogenesis of airway dysfunction in equine COPD is beyond the scope of the current study. However, these data do suggest that the residence of neutrophils in the airways (approximately 4 days) may be temporally associated with the duration of

airway hyperresponsiveness (at least 3 days; Fairbairn *et al.* 1993a, but less than 7 days; Derksen *et al.* 1985a) induced by a single short-term hay/straw challenge. The associations between inflammatory cell function and airway dysfunction are complex. If their effects were solely related to release of typically short-lived histotoxic agents, correlations between airway dysfunction and the presence of neutrophils or their secretory products might be more easily appreciated. Not only can granulocytes also generate lipid mediators and chemokines, with the ability to both recruit other inflammatory cells and activate resident airway cells, but the action of histotoxic neutrophil products on host tissues may produce further pro-inflammatory molecules. This interplay leads to amplification of the inflammatory changes beyond those caused by the any single cell type or mediator alone (reviewed in Sections 1.3.3, 1.6 and 1.7). Thus proof of a direct causative relationship between inflammation and airway dysfunction continues to elude clinical researchers even in the investigation of important human diseases such as asthma (Haley and Drazen, 1998).

CHAPTER 8

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CHAPTER 9

PUBLICATIONS

9.1 PAPERS

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Priming induces functional coupling of *N*-formyl-methionyl-leucyl-phenylalanine receptors in equine neutrophils

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Abstract: The synthetic formylpeptide fMLP is widely used as a model chemoattractant and secretagogue for mammalian neutrophils. Despite possessing fMLP receptors, equine neutrophils do not produce superoxide anions in response to fMLP and there is no inflammatory reaction in the horse when fMLP is injected intradermally. The functional capability of these receptors was investigated after pretreatment with recognized priming agents. Purified neutrophils were pretreated with lipopolysaccharide (LPS), platelet-activating factor (PAF), or tumor necrosis factor α (TNF-α) and superoxide anion generation and shape change quantified by lucigenin-dependent chemiluminescence (LDCL) and flow cytometry, respectively. LPS, TNF-α, and PAF pretreatment induced significant LDCL in response to fMLP; similarly LPS pretreatment was a prerequisite for fMLP-stimulated neutrophil polarization in response to fMLP. However, LPS failed to induce fMLP-mediated chemotaxis of equine neutrophils. These data indicate that equine neutrophil fMLP receptors are not vestigial as previously thought but can trigger both respiratory burst activity and cell polarization responses after priming. *J. Leukoc. Biol.* 63: 380–388; 1998.

Key Words: *horse · lipopolysaccharide · priming*

INTRODUCTION

Neutrophils play a pivotal role in the acute cellular response to infection and injury and have evolved a series of unique functions that facilitate both their recruitment to sites of inflammation and their ability to destroy invading microorganisms [1–4]. During the inflammatory process, however, inappropriate or excessive secretion of histotoxic or proinflammatory substances by the neutrophil can contribute to host tissue damage [1–4], and this sequence of events is also recognized in the horse [5, 6]. In many species the responsiveness of granulocytes to secretagogue stimuli is governed by prior exposure to priming agents such as bacterial products [e.g., lipopolysaccharide (LPS) and *N*-formylated peptides], cytokines [e.g., tumor necrosis factor α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF)], and lipid-derived mediators [e.g., platelet-activating factor (PAF) and leukotriene B_4]. Such agents sensitize the neutrophil to enhance certain functional responses, e.g., superoxide anion generation, adhesion, and proteolytic enzyme release when subsequently exposed to secretagogue agonists [7–12]. In addition, neutrophil priming is thought to be crucial to the initial recruitment of these cells to an inflamed site [13] and appears to be a prerequisite for neutrophil-mediated host tissue damage [14, 15].

The synthetic tripeptide, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), which mimics the effects of a number of bacterial cell wall-derived peptides, is recognized by specific high-affinity receptors on the neutrophil [16] and has been widely used as a model agonist for studying the response of mammalian neutrophils to bacterial infection, sepsis, and tissue injury [16]. The large and consistent nature of the secretory and chemotactic response induced by fMLP in human cells has also made it the agent of choice in studies exploring the mechanisms underlying priming and activation. However, despite possessing a small number of seemingly identical high-affinity fMLP receptors [17], equine neutrophils appear to display a very different pattern of responses to this agent, to the extent that most recent reports have regarded the equine fMLP receptor as being of little, if any, functional significance [18–20]. This conclusion is supported by the lack of any inflammatory response when fMLP is injected intradermally in the horse even at concentrations as high as 10 mM per 100 μL

Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; PAF, platelet-activating factor; TNF-α, tumor necrosis factor α; LDCL, lucigenin-dependent chemiluminescence; GM-CSF, granulocyte-macrophage colony-stimulating factor; LBP, LPS-binding protein; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; ZAP, zymosan-activated plasma.

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injection volume [21]. Despite this, a small and selective number of early in vitro studies reported fMLP effects in equine neutrophils, including induction of chemotaxis, albeit only at very high fMLP concentrations [22], and lysosomal enzyme release [17]. Although Snyderman and Goetzl [23] stated that fMLP could induce superoxide anion generation in equine neutrophils, no experimental data were provided to support this. Hence, although inter-species differences have been demonstrated in studies of inflammation in the horse [20, 24], the precise function and role of the fMLP receptor in equine neutrophils remains uncertain. In view of both the importance of bacterial endotoxemia to the pathogenesis of many equine diseases [4, 5, 25], and the relative lack of information regarding the effects of LPS on equine neutrophils, we have chosen to examine the functional effects of this and other human granulocyte priming agents on the equine neutrophil. These studies (1) provide clear evidence of fMLP-receptor-mediated superoxide anion generation and cell polarization in equine neutrophils after LPS incubation and (2) suggest that the underlying mechanism for LPS priming in these cells is similar to that documented in other mammalian cells because the sensitivity to LPS is enhanced in the presence of serum and hence likely to be mediated by the LPS-binding protein (LBP)/CD14 pathway. This study permits a greater understanding of the interaction of LPS with equine neutrophils and indicates the potential for fMLP-like peptides to induce equine granulocyte activation via functional fMLP receptors. This model should also permit assessment of the importance of neutrophil priming to the recruitment and function of these cells at inflamed sites.

MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA), Dextran T500, fMLP, glutaraldehyde, LPS from *Escherichia coli* serotype 0111:B4, Hanks' balanced salt solution (HBSS), lucigenin (bis-*N*-methylacridinium nitrate), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and phosphate-buffered saline with (PBS) or without (PBS w/o) 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, PAF, and zymosan were purchased from Sigma (Poole, Dorset, UK). Sodium citrate (3.8%) was purchased from Phoenix Pharmaceuticals Ltd. (Gloucester, UK) and the silicone oil F-50 was obtained from Croylek Ltd. (Surrey, UK). TNF-α was purchased from Genzyme, (Cambridge, MA). Percoll was obtained from Pharmacia (Uppsala, Sweden). [^{3}H]fMLP [specific activity 1.48–3.219 TBq (40–87 Ci)/mmol] was purchased from New England Nuclear Life Science Products (Stevenage, Herts, UK). Diff-Quik was purchased from B. M. Brown Ltd. (Reading, Berks, UK). All other chemicals were of molecular or reagent grade and were obtained from BDH (Leicestershire, UK). Autologous heat-inactivated serum was prepared by the addition of 220 µL of 10 mM $CaCl_2$ to 10 mL of platelet-rich plasma, incubating for 90 min at 37°C in a humidified 5% CO_2 atmosphere, then heating at 56°C for 30 min. Zymosan-activated plasma (ZAP) was used as a source of the complement fragment C5a and was produced by adding 5 mg/mL zymosan to platelet poor plasma (PPP), followed by sonication and incubation at 37°C for 60 min. The suspension was centrifuged at 1,400 *g* for 15 min. The supernatant was removed and stored in 100 µL aliquots at −20°C until required. ZAP was used in all experiments at a final concentration of 10% (v/v).

Preparation of equine neutrophils

Blood was drawn from healthy adult horses by jugular venipuncture and anticoagulated with 1 mL of 3.8% sodium citrate per 10 mL of blood in 50-mL sterile polyethylene tubes. Thereafter three different methods [8, 26, 27] for isolating equine neutrophils were compared. The methods of Jain et al. [26] and Pycock et al. [27] both employed a single-step isolation technique with discontinuous isotonic Percoll gradients (59 + 75 and 70 + 85% Percoll in HBSS, respectively) and were used as originally detailed. The third technique was a modification of a method originally described for isolating human neutrophils which uses discontinuous hypotonic plasma/Percoll gradients [8]. In this method, whole blood was allowed to sediment under gravity for 30 min at room temperature and the upper leukocyte-rich plasma layer aspirated and centrifuged at 367 *g* for 6 min. The platelet-rich plasma supernatant was decanted and centrifuged at 2400 *g* for 20 min to prepare PPP. The initial leukocyte-rich pellet, obtained typically from 80 mL of whole blood, was resuspended in 2 mL PPP in a 15-mL polystyrene tube and underlayered with 42 and 51% PPP/Percoll solutions before centrifugation at 255 *g* for 10 min. Cytospins prepared after gradient centrifugation indicated that the majority of the neutrophils accumulated in a tight band at the 42/51% Percoll interface with the mononuclear cells held up at the upper, PPP/Percoll, interface. The very small number of eosinophils present (2.5 ± 0.26% of total leukocyte yield) settled in either the 51% Percoll layer or with the erythrocyte pellet. Neutrophils were harvested from the 42/51% interface and washed sequentially in PPP, PBS without Ca^{2+} Mg^{2+}/0.1% BSA, and PBS before resuspension in PBS. Subsequent experiments indicated that a more extensive wash protocol (three extra washes in PBS without Ca^{2+} Mg^{2+}/0.1% BSA) was necessary to reveal the true serum dependence of the mechanism of neutrophil priming by LPS in equine neutrophils. All the procedures outlined were performed at room temperature. Cell purity was assessed using cytocentrifuge preparations stained with Diff-Quik; cell viability was assessed by trypan blue exclusion. In a limited number of studies, human neutrophils were also employed and these were prepared in an identical manner to that detailed above, with the exception that citrated venous blood was allowed to sediment with 6% dextran T500 to obtain the initial leukocyte-rich layer [8].

Superoxide anion generation

Neutrophils were resuspended at 12.5 × 10^6 cells/mL in PBS and incubated in a shaking waterbath at 37°C for 5 min before stimulation at predetermined optimal times, with LPS (0.1–1000 ng/mL ± 1% serum for 30, 60, or 90 min), PAF (100 nM, 10 min), or TNF-α (200 U/mL, 30 min). Basal and agonist-stimulated superoxide anion generation was measured by lucigenin-dependent chemiluminescence (LDCL) using a ML 3000 microtiter plate luminometer (Dynatech Laboratories Ltd., Billingshurst, West Sussex, UK). Lucigenin (0.25 mM in PBS containing 1 mg/mL BSA) was added (100 µL) to each well and allowed to equilibrate at 37°C for 15 min before the addition of 80 µL (1 × 10^6) of freshly isolated cells. After a 5-min equilibration period, basal LDCL was recorded over 12 min to ensure that the cells were not basally activated (stable basal peak LDCL <0.005 RLU). After preincubation, 20 µL of buffer or fMLP was added to triplicate wells and chemiluminescence recorded at 8-s intervals for 5 min. This time course was extended to 60 min in experiments using ZAP. Data were recorded and analyzed on-line (Cellular Chemiluminescence, Dynatech Laboratories Ltd.) to produce mean peak and integral chemiluminescence values from triplicate wells. All chemiluminescence data are presented as integral (area under the curve) values unless otherwise stated.

Neutrophil shape change

Neutrophils (2 × 10^6 in 900 µL PBS) were incubated at 37°C in the presence or absence of LPS (1 µg/mL) for 90 min in a shaking waterbath. Duplicate samples were then incubated for 10 min with 100 µL buffer or 100 nM fMLP. Incubations were terminated by the addition of an equal volume of 2.5% glutaraldehyde. Shape change was quantified by flow cytometry (EPICS Profile II, Coulter Electronics, Luton, Bedfordshire, UK) using a minor modification of the method described by Cole et al. [28] as previously detailed [29]. Values obtained for shape change using this analysis method correlate closely with those obtained by direct light microscopic assessment of shape change, with the exception that the flow cytometric method of assessment slightly overestimates the extent of basal shape change [28].

Neutrophil chemotaxis

Neutrophils resuspended in PBS (3 × 10^6 cells/mL) were incubated with 1% serum for 90 min in the presence or absence of LPS (final concentration 1

TABLE 1. Comparison of Three Different Methods for Isolating Equine Neutrophils from Peripheral Blood

Isolation method	Cell purity (% ± SEM)	Cell viability (% ± SEM)	Cell recovery (% ± SEM)
Jain et al., 1990 [25]	96.3 ± 2.4	98.0 ± 0.5	26.5 ± 2.6
Pycock et al., 1987 [26]	98.7 ± 0.6	97.7 ± 0.9	35.0 ± 4.7
Haslett et al., 1985 [8]	98.2 ± 0.3	99.3 ± 0.1	43.8 ± 2.6

Three previously published techniques for preparing equine [26, 27] and human [8] neutrophils were compared. Cell purity was assessed morphologically on fixed and stained cytospin preparations, cell viability by trypan blue exclusion, and cell recovery by comparison of final yields measured using a hemocytometer to automated Coulter counting of whole blood samples. Data represent mean ± SEM of n = 4–15 separate experiments.

μg/mL) and chemotaxis assayed in a Neuroprobe 96-well chemotaxis chamber (Porvair Filtronics Ltd.) incorporating a 5-μm pore size polycarbonate filter. PBS (control), fMLP (0.01–1000 nM), or ZAP (10% v/v) were placed into the lower wells (35 μL) and 225 μL of buffer or LPS-pretreated neutrophils added to the upper wells. The chamber was incubated in a humidified 5% CO_2 atmosphere at 37°C for 90 min. The filter was then removed, the upper surface scraped with a cell scraper, washed with 0.9% saline, air dried, fixed, and stained with Diff-Quik. Cell migration into, and retention within, the filter was quantified by measuring the optical density of each well footprint at 500 nm in a MR 5000 plate reader (Dynatech Laboratories Ltd., Billingshurst, UK).

[^{3}H]fMLP binding to equine neutrophils

The [^{3}H]fMLP binding assay was based on the method described by O'Flaherty et al. [30]. In brief, neutrophils (12.5×10^6/mL) were pre-incubated for 90 min in PBS plus 1% serum in the presence or absence of 1 μg/mL LPS then diluted with an equal volume of ice-cold PBS containing 10 mM HEPES (pH 7.4), centrifuged at 235 g for 6 min and resuspended at 5.26×10^6 cells/mL. [^{3}H]fMLP binding was performed by incubating 5×10^6 cells in the above buffer at 4°C for 60 min with 0.0632, 0.2, 0.632, or 2 nM [^{3}H]fMLP alone or 2 nM [^{3}H]fMLP with sufficient fMLP to make up final ligand concentrations of 0.0632–6320 nM in half log dilution steps. Incubations were terminated by centrifugation (10,000 g, 2 min, 4°C) through a 400-μL silicone oil cushion. Aliquots (200 μL) of the supernatant and the isolated cell pellets were transferred to scintillation vials; the pellets were incubated with 500 μL methanol for 15 min and 4 mL of scintillation fluid was added to each sample before scintillation counting. Data underwent Scatchard analysis using LIGAND software [31], where the number of fMLP molecules bound per neutrophil was calculated using Avogadro's constant and the picomoles of fMLP bound per 5×10^6 cells.

Statistics

Results were expressed as means ± SEM values for (n) sets of separate experiments. Data were analyzed using analysis of variance followed by the Student-Newman-Keuls post test or by the Student's paired t test. Results were considered to be significant with P values < 0.05. EC_{50} values were obtained by analysis using Kaleidagraph (Macintosh) software.

RESULTS

Comparison of different methods for purifying equine neutrophils

Table 1 shows the cell purity, viability, and recovery rates for equine neutrophils isolated using the three different Percoll gradient methods outlined. The method of Haslett and coworkers [8] generated cells of consistent and high purity (98.2 ± 0.3%) and superior cell viability and recovery compared to the methods of Jain et al. [25] and Pycock et al. [26]. This method was therefore chosen as the technique of choice for the subsequent experiments. The method of Haslett et al. [8] has also been shown to induce minimal priming and shape change of human neutrophils and, in 12 sequential neutrophil isolations from different horses, basal shape change assessed by light microscopy was confirmed to be extremely low at 2.9 ± 1.3%.

Induction of fMLP-induced superoxide anion generation by neutrophil priming agents

Preliminary experiments were designed to characterize C5a-stimulated LDCL in freshly isolated equine neutrophils to facilitate subsequent assessment of fMLP-stimulated effects. ZAP, a biological source of C5a, stimulated a large LDCL signal (24.3 ± 5.8 RLU, n = 8), with the peak of superoxide anion generation occurring between 20 and 25 min post-stimulation (**Fig. 1**). The effect of ZAP was significantly enhanced by pretreatment with 100 ng/mL LPS (147 ± 10%, n = 8, $P <$ 0.01, Fig. 1). In contrast, incubation of neutrophils with 1 μM fMLP did not stimulate superoxide anion release compared with buffer-treated control cells (control, 0.2 ± 0.04 RLU; fMLP, 0.3 ± 0.1 RLU; n = 17, $P > 0.05$). After LPS priming, however, a significant and rapid fMLP-induced respiratory burst response was apparent (LPS + buffer, 0.4 ± 0.1 RLU; LPS + fMLP, 2.4 ± 0.1 RLU, n = 8, $P < 0.05$, Fig. 1), although this was less marked than that obtained with ZAP. Due to the fast kinetics of this response, a more detailed time course was examined (**Fig. 2**). This demonstrated a peak of superoxide anion generation at 2 min after fMLP stimulation of LPS-primed cells and again a lack of effect of fMLP in cells preincubated with buffer alone. The optimum conditions for LPS priming were then investigated.

The effect of LPS on fMLP-stimulated LDCL was observed to

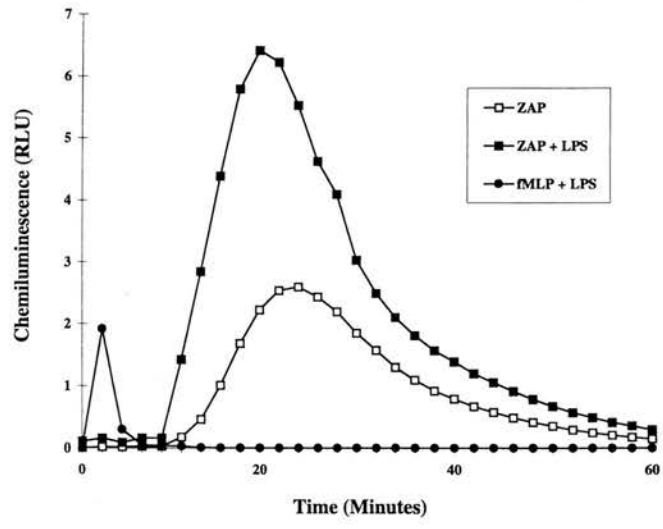

Fig. 1. Effect of LPS on ZAP- and fMLP-induced superoxide anion generation. Equine neutrophils were incubated with PBS or 100 ng/mL LPS + 1% serum for 90 min before addition of lucigenin and stimulation with PBS, 10% v/v ZAP, or 100 nM fMLP. Lucigenin-dependent chemiluminescence (LDCL) was monitored over a 60-min time period using a ML3000 microtiter plate luminometer and expressed in relative light units (RLU). The LDCL signal generated by PBS or LPS pretreated cells stimulated with PBS, and PBS pretreated cells stimulated with fMLP was not significantly different to baseline and hence these values have been omitted for clarity. Values represent means of triplicate measurements from a single experiment representative of eight.

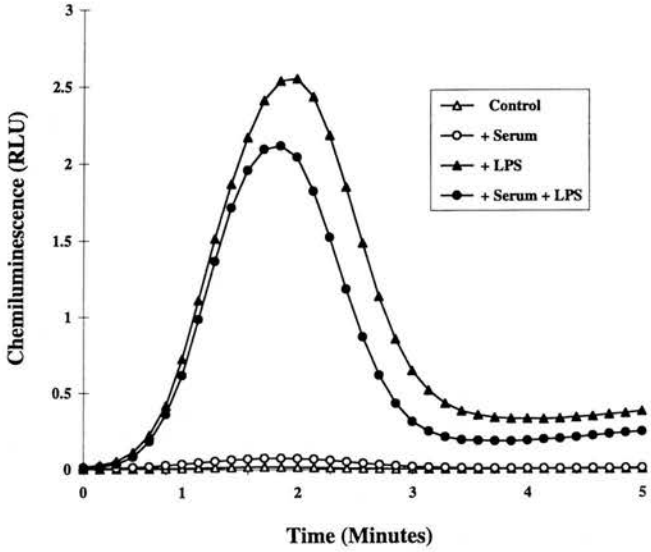

Fig. 2. Time course of fMLP-stimulated superoxide anion generation. Time-course of fMLP-stimulated LDCL after a 90-min pretreatment with PBS or 1 μg/mL LPS both performed in the presence and absence of 1% serum. Values represent means ± SEM of triplicate measurements from three separate experiments. Error bars omitted for clarity; SEM values were <10%.

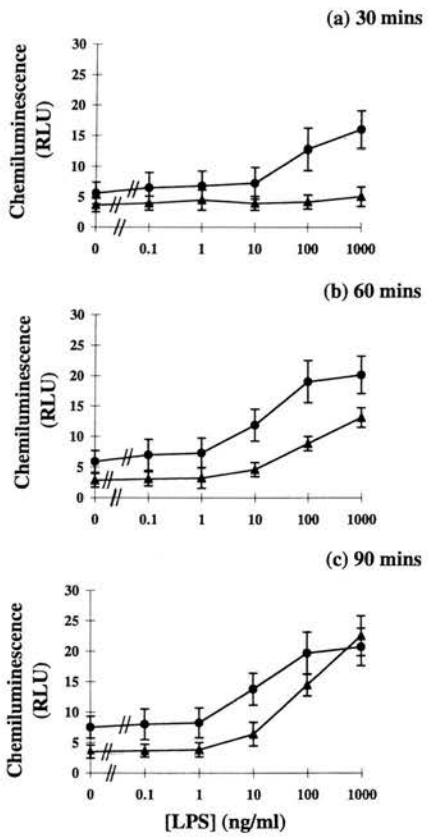

Fig. 3. Characterization of LPS enhancement of fMLP-stimulated superoxide anion generation. LPS concentration-response curves for fMLP (1 μM) -stimulated LDCL after incubation with buffer or LPS (0.1–1000 ng/mL) in the presence (circles) or absence (triangles) of 1% equine serum for (A) 30, (B) 60, or (C) 90 min. Values represent means ± SEM of five separate experiments.

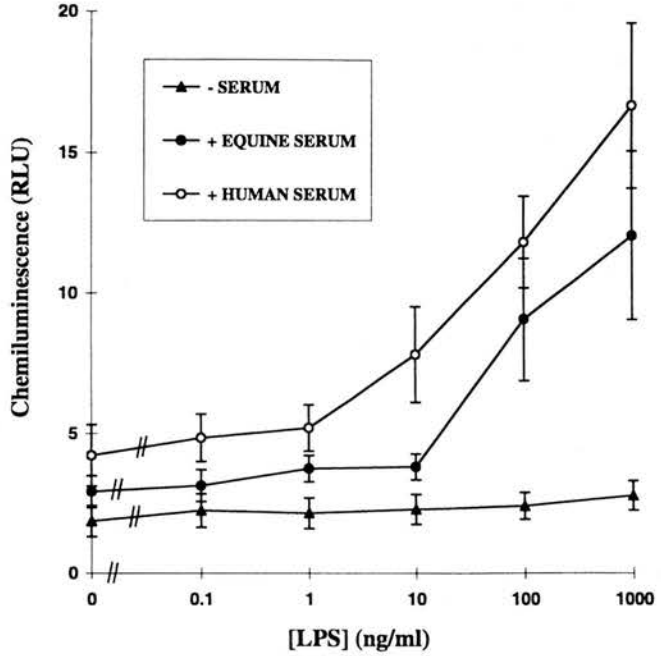

Fig. 4. Human serum also enhances LPS priming of equine neutrophils. LPS concentration-response curves for 1 μM fMLP-stimulated LDCL in equine neutrophils after a 30-min incubation with LPS (0.1–1000 ng/mL) in the presence and absence of equine or human serum. Values represent means ± SEM of three separate experiments.

be both time- and concentration-dependent (**Fig. 3, A–C**). Addition of 1% serum significantly potentiated both the temporal initiation of the primed fMLP response and the cells' sensitivity to LPS (Fig. 3, A–C). In the presence of serum the LPS-primed response was maximal by 60 min with a mean EC_{50} of 19.1 ± 4.7 ng/mL. However, in the absence of serum, a 90-min LPS incubation was required to achieve an equivalent maximal response, albeit with the cells remaining less sensitive to LPS (Fig. 3, A–C). It is interesting to note that the true serum dependence of this effect in equine neutrophils was only revealed after an extensive initial wash protocol, suggesting that LBP or its equine equivalent is tightly adherent to equine neutrophils.

LPS priming for enhanced superoxide anion generation in human neutrophils is well recognized to be largely serum dependent due to a requirement for LBP [32, 33] and these data would suggest the involvement of a similar LBP/CD14 mechanism for LPS priming in equine neutrophils. Indeed we show that human serum can substitute for equine serum in potentiating the LPS priming of equine neutrophils (**Fig. 4**) and vice versa (data not shown). The fMLP effect on superoxide anion generation in LPS-primed neutrophils was also shown to be concentration dependent with an EC_{50} of 10.2 ± 3.9 nM (**Fig. 5**).

To examine whether LPS was unique in its ability to induce functional coupling of the fMLP receptor in equine cells, the effects of PAF and TNF-α were also investigated. Although, compared to its effects in human cells [29], PAF appeared to have a very weak priming effect, TNF-α induced a major up-regulation of fMLP-induced LDCL (**Fig. 6**). However, TNF-α treatment alone also stimulated a significant LDCL response, indicating that this cytokine was acting as a secretagogue in addition to its priming action (Fig. 6).

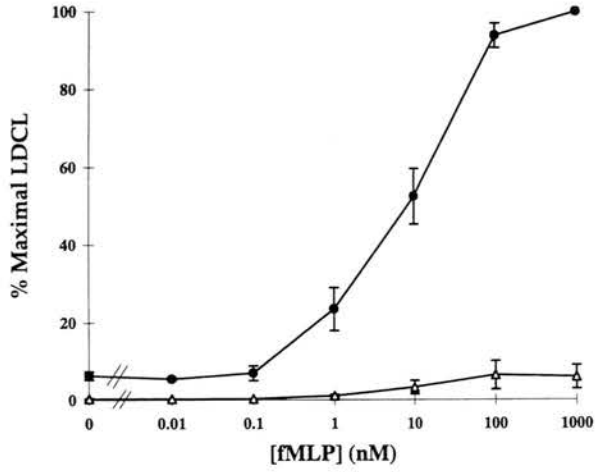

Fig. 5. Concentration-response curve for fMLP-stimulated superoxide generation in LPS-primed equine neutrophils. Equine neutrophils were pre-incubated with PBS (open triangles) or 1 μg/mL LPS + 1% serum (filled circles) for 90 min before addition of lucigenin and stimulation with 0.01–1000 nM fMLP. LDCL was determined over a 5-min period. Data represent mean ± SEM of triplicate determinations from a single experiment representative of three (mean maximal LDCL 9.9 ± 0.6 RLU).

Induction of fMLP-induced neutrophil shape change by LPS

In contrast to the effects of ZAP (10%, v/v), which caused a dramatic shape change response (96.3 ± 0.7% cells shape changed, 10 min), freshly isolated equine neutrophils did not polarize when exposed to 1 μM fMLP for the same period of time (% shape change: control, 9.0 ± 1.5%; fMLP, 9.9 ± 1.9; $n = 4$, $P > 0.05$). Likewise, incubation of cells with buffer alone for 90 min did not influence this lack of response to fMLP (% shape change: control, 12.9 ± 1.5%; fMLP, 16.8 ± 2.1; $n = 4$, $P > 0.05$, **Fig. 7**). However, after treatment of cells with 1 μg/mL LPS for 90 min, fMLP induced a significant increase in equine neutrophil shape change (% shape change: LPS 14.5 ± 3.8%; LPS + 1 μM fMLP, 29.4 ± 5.0%; $n = 4$, $P < 0.05$, Fig. 7) with an EC_{50} for fMLP of 1.9 ± 0.9 nM (data not shown). The inability of fMLP to induce cell polarization in unprimed cells is in marked contrast to its effects in human neutrophils, which undergo a rapid and concentration-dependent shape change response to this agent alone [29] (Fig. 7).

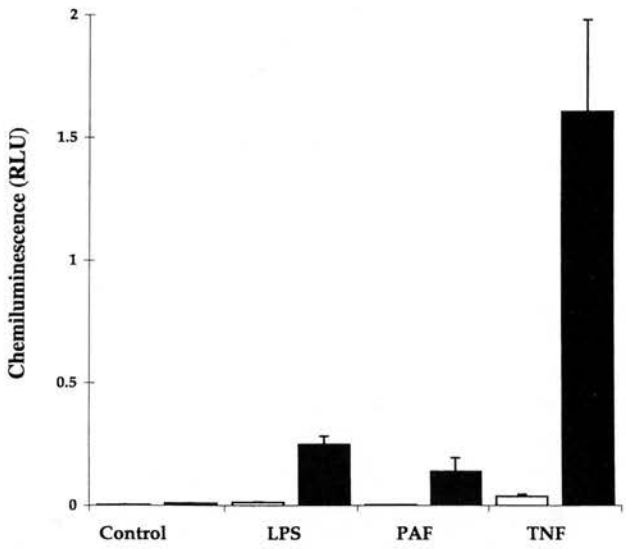

Fig. 6. Priming of fMLP-stimulated superoxide anion generation by LPS, PAF, and TNF-α in equine neutrophils. Equine neutrophils were pre-incubated with LPS + 1% serum (1 μg/mL, 90 min), PAF (100 nM, 10 min), TNF-α (200 U/mL, 30 min), or PBS (90 min) before addition of lucigenin and stimulation with PBS (open bars, left of pair) or 100 nM fMLP (filled bars, right of pair). LDCL was recorded at 8-s intervals for 5 min. Peak values presented as means ± SEM of three separate experiments, each performed in triplicate.

LPS fails to induce fMLP-mediated chemotaxis in equine neutrophils

Analysis of chemotaxis data failed to demonstrate any chemotactic response of unprimed equine neutrophils to 0.01–1000 nM fMLP (**Fig. 8**). In contrast to the above superoxide anion generation and cell polarization data, pretreatment of equine neutrophils with 1 μg/mL LPS for 90 min did not permit fMLP-directed chemotaxis (Fig. 8). Positive control chemotaxis data were obtained both in equine cells using ZAP (Fig. 8) and human cells using fMLP (data not shown).

[^{3}H]fMLP radioligand binding studies

To determine that the effect of fMLP was likely to be receptor-mediated, radioligand binding studies were undertaken to quantify fMLP receptor number and affinity in equine neutrophils and to examine the effects of LPS priming on receptor expression.

Scatchard analysis of [^{3}H]fMLP binding to equine neutrophils demonstrated a single set of high-affinity fMLP receptors (K_d 9.3×10^{-11} M), with a mean of 660 ± 159 ($n = 3$) receptors per cell (**Fig. 9**). This compares to data obtained in parallel experiments in human neutrophils where Scatchard analysis demonstrated a two-receptor site model with a mean of 36,000 low-affinity receptors (K_d 3.2×10^{-8} M) and 2,100 high-affinity receptors (K_d 9.2×10^{-10} M) per cell ($n = 3$), consistent with previous reports [30]. After LPS priming (1 μg/mL, 90 min) of equine cells, a similar single receptor site model fit was again obtained (K_d 1.4×10^{-10} M), with a significant ($P < 0.05$) 2.5-fold increase in total receptor numbers to 1,400 ± 398 per cell ($n = 3$; Fig. 9).

DISCUSSION

Although several methods for the isolation of neutrophils from equine peripheral blood have been described [26, 27], no comparison of these methods has been reported. The importance of employing a neutrophil isolation technique that produces a highly purified population of minimally primed cells has been established as a crucial factor in studying neutrophil natural biology in humans [8] but this factor has not been previously addressed in equine leukocytes. The very high erythrocyte sedimentation rate in the horse obviates the need for the initial cell centrifugation and dextran sedimentation steps required for the preparation of human cells. With this modification, the method of Haslett et al. [8] allowed isolation of a population of highly purified cells with minimal basal shape change and LDCL generation, and a superior cell yield

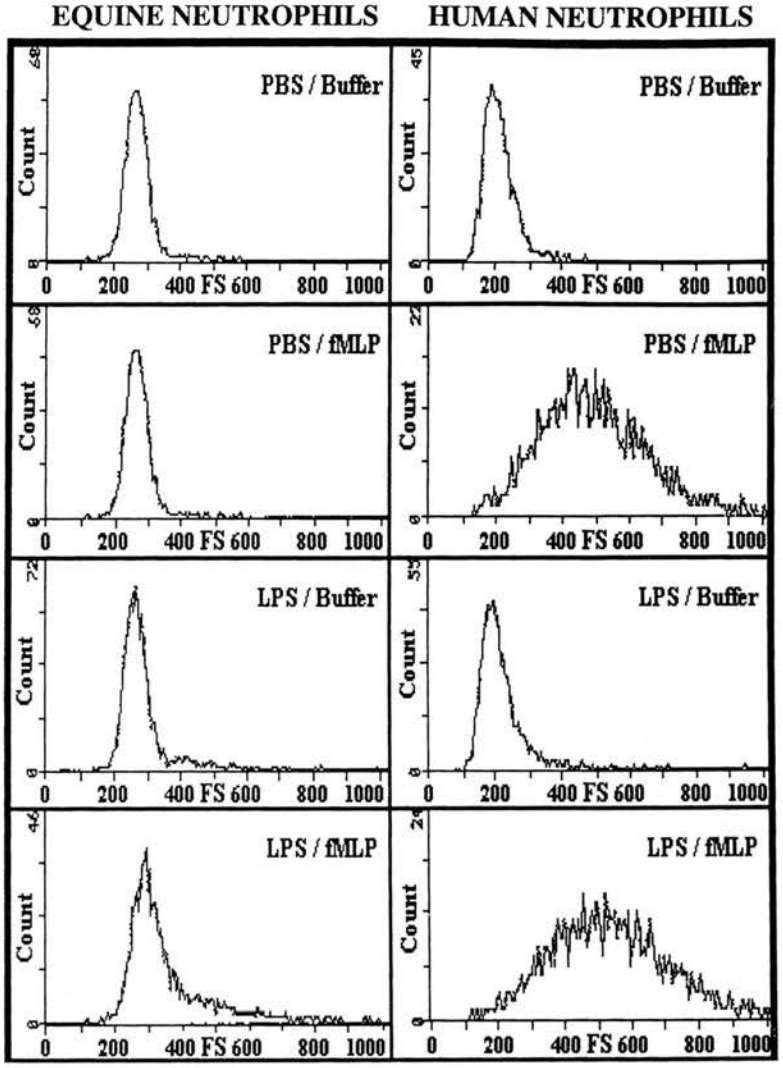

Fig. 7. Effect of LPS on fMLP-induced shape change. Equine or human neutrophils were pre-incubated for 90 min with PBS or 1 μg/mL LPS before stimulation with buffer or 100 nM fMLP for 10 min. Representative flow cytometry histograms of glutaraldehyde-fixed equine (left panels) and human (right panels) neutrophils are shown (pre-incubation/stimulation conditions are indicated within the panels). Data represent mean forward light scatter (*x*-axis) plotted against cell number (*y*-axis). Similar data were obtained from three additional experiments, each performed in duplicate.

compared to the two isotonic HBSS/Percoll gradient methods used. The high cell purity obtained is aided by the relatively low numbers of eosinophils present in equine blood. Although the extent of basal activation and/or priming was not directly compared between the three isolation methods used, the high viability values recorded for all three techniques and the extremely low basal shape change and LDCL values obtained for the plasma/Percoll isolated cells suggest that basal activation was minimal for all three preparative methods.

Lucigenin-enhanced chemiluminescence (LDCL) has been

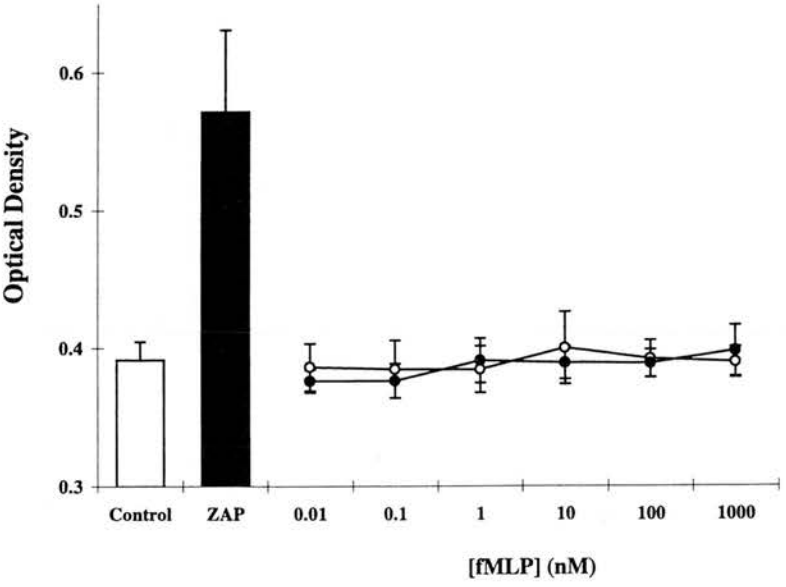

Fig. 8. Inability of LPS to induce fMLP-stimulated chemotaxis. Equine neutrophils were incubated for 90 min in PBS + 1% serum in the presence (filled circles) or absence (open circles) of 1 μg/mL LPS. Chemotaxis toward fMLP (0.01–1000 nM) or ZAP (10% v/v) was assayed using a Neuroprobe 96-well chemotaxis chamber incorporating a 5-μm pore filter that was stained with Diff-Quick before measurement of optical density. Data represent mean ± SEM of five separate experiments, each performed in triplicate.

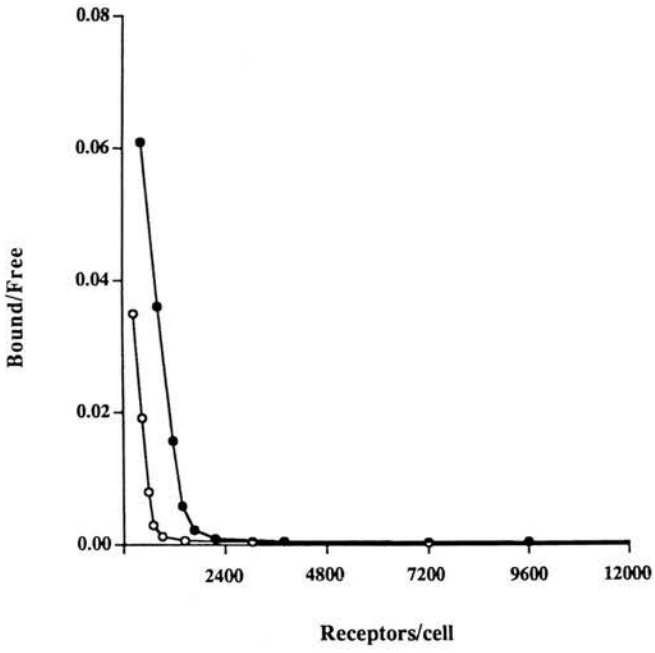

Fig. 9. Scatchard analysis of [^{3}H]fMLP binding to equine neutrophils. Equine neutrophils were pre-incubated in bulk for 90 min in PBS plus 1% autologous serum in the presence (filled circles) or absence (open circles) of 1 μg/mL LPS before assessment of [^{3}H]fMLP binding as detailed in Materials and Methods. Scatchard transformed data is shown where each point represents the mean of three experiments each performed in triplicate.

used widely to study oxidative metabolism during phagocyte respiratory burst activity [34, 35]. It is independent of myeloperoxidase [34] and has been demonstrated to provide a sensitive and selective measurement of extracellular superoxide anion release [36]. Previous studies examining superoxide anion generation in equine neutrophils using superoxide dismutase-inhibitable reduction of cytochrome *c* suggested that freshly isolated equine cells were unresponsive to fMLP. In light of the absent, extremely weak, or inconsistent in vitro [17–19, 22, 35] and in vivo [21] responses elicited by fMLP in the horse, the lack of effect of fMLP on superoxide anion release in this species was initially attributed to a lack of any fMLP receptor coupling. Indeed we were unable to detect any chemotactic or polarization response to fMLP in unprimed equine neutrophils despite the very clear mobility effects of this agent in human neutrophils, which are observed even in fully unprimed cells [29, 37, 38]. However, previous data demonstrating a concentration-dependent induction of lysosomal enzyme release by fMLP [17] suggested that functional coupling of fMLP receptors might be possible in the horse and that superoxide anions might be produced under appropriate conditions because these two effects usually occur concurrently [8].

Our model was specifically developed for its high sensitivity for detecting superoxide anion release from cells [34] and demonstrated that fMLP-stimulated LDCL could indeed be observed, but this only occurred to any significant extent after LPS pretreatment, i.e. consistent with this being a primed response [7, 8]. A similar effect was also observed with PAF and TNF-α, both well-recognized neutrophil priming agents in other species [29]. Furthermore, the priming effect of LPS was not restricted to fMLP, since, in agreement with a previous report [11], ZAP-stimulated LDCL was also enhanced by LPS pretreatment. The kinetics of fMLP-induced LDCL in LPS-primed equine neutrophils demonstrated an extremely brisk superoxide anion response with significant LDCL observable 30 s post-fMLP addition with peak LDCL occurring between 60 and 120 s. Thereafter, respiratory burst activity abated rapidly with virtually complete desensitization of the response by 4 min. This pattern of superoxide generation is very similar to that reported for human cells [34, 36].

The induction of fMLP-induced shape change following priming was also of interest, not least because a very brisk polarization response is observed in human neutrophils and this is not dependent on priming status [29]. In human cells, however, LPS exposure has been shown to enhance the rate and maximal extent, and reduce the lag time, of F-actin polymerization induced by fMLP [39]. In equine cells, the pre-incubation conditions necessary for LPS-induced priming for enhanced fMLP LDCL responses did not produce significant cell shape-change with LPS alone and hence in this species, unlike in humans [8], measurement of cell shape change alone rather than more complex cytoskeletal changes may not be the most appropriate or indeed accurate method of detecting cell priming or of modeling the control of cytoskeletal elements in vitro. This necessity for LPS priming to facilitate equine neutrophil shape change by functional fMLP receptor-ligand coupling could provide an important and novel model to dissect signal transduction pathways linking priming responses to cell polarization.

Detailed characterization of the priming effect of LPS in equine cells demonstrated that enhancement of the fMLP-stimulated LDCL response, although requiring slightly higher LPS concentrations (≥10 ng/mL) than human cells [7, 10, 31] evolved over a very similar time course. The ability of serum to enhance the responsiveness of leukocytes from other species to LPS [32, 33, 41], reflects the formation of a complex between LPS and a serum LBP that is recognized by the glycosyl-phosphatidylinositol-anchored membrane protein, CD14 [42]. These data provide strong evidence for a similar paradigm underlying the mechanism of LPS interaction with equine neutrophils. The presence of LBP has not previously been studied in the horse and consequently appropriate reagents for its quantitative analysis are not available. However, the ability of human and equine serum to substitute for one another in these experiments suggests that a functional homologue of LBP is indeed present in equine serum. The currently available anti-human CD14 monoclonal antibodies do not bind to equine neutrophils [T. J. Brazil, unpublished data] and hence the presence or absence of CD14 on these cells and a more detailed dissection of this mechanism, remains to be explored in this species.

The chemotaxis data presented are in agreement with most previous reports in equine neutrophils [17, 18]. However, Zinkl et al. [22] and Sedgwick et al. [19] observed a chemotactic effect of fMLP but only at high, non-physiological, concentrations, i.e. 100 μM; all other groups have failed to observe any chemotactic response to this agonist [17, 18]. Taken together,

these data suggest that fMLP is at best a very poor chemotactic agent for equine neutrophils and that LPS exposure is unable to modulate this lack of response. Of note, LPS exposure actually reduces the chemotactic responsiveness of human neutrophils to fMLP [8], however, the response of equine neutrophils to ZAP in our system was unaffected by LPS (data not shown).

Scatchard analysis of [^{3}H]fMLP binding to equine neutrophils was able to confirm the presence of specific fMLP binding sites in equine neutrophils and also identified a 2.5-fold increase in fMLP receptor number after LPS priming without altering the single receptor site model fit or changing receptor affinity. A similar single site model for equine neutrophils was described by Snyderman and Pike [17] in which they identified 630 $\pm$ 184 receptors per cell equivalent to the number we found in unprimed cells. A single fMLP receptor site has also been identified in rabbit neutrophils where receptor expression was increased eightfold after intravenous endotoxin administration [43]. However, this was not accompanied by any comparable effect on neutrophil function, suggesting that the two effects were not directly related [44]. This point is reinforced by studies in human neutrophils that have demonstrated that the increase in fMLP receptor expression observed in response to priming [30, 45] or bacterial infection [46] either lags behind [30] or does not correlate with [45, 46], the enhanced functional response but results from translocation of preformed fMLP receptors to the plasma membrane, which occurs as a consequence of fusion of specific granules with the cell surface [47]. The possibility that the small change in fMLP receptor number identified in equine cells post-LPS treatment is a consequence, rather than a cause, of priming, is supported by the observation that receptor numbers for other human neutrophil agonists (e.g., C5a), whose responses are equally enhanced, are not affected by LPS [45]. It is unlikely therefore, that changes in receptor number alone represent a major control point determining the magnitude of the fMLP receptor-mediated response. Hence, the dramatic augmentation of the fMLP response observed most likely occurs at a level downstream from ligand-receptor binding in the signal transduction cascade. Although further studies are required to dissect both the precise mechanism of LPS interaction with equine neutrophils and the subsequent intracellular signaling steps that stimulate functional coupling of the fMLP receptors in these cells, the very dramatic and selective priming response observed in the horse neutrophil may make this cell an ideal, and hitherto unrecognized, model for the investigation of functional coupling of leukocyte G-protein-linked chemoattractant receptors.

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Role of neutrophil apoptosis in the resolution of pulmonary inflammation

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ABSTRACT: *Role of neutrophil apoptosis in the resolution of pulmonary inflammation. K. Mecklenburgh, J. Murray, T. Brazil, C. Ward, A.G. Rossi, E.R. Chilvers.*

Neutrophil-mediated lung injury may result from one or more of the following possible causes: 1) loss of the normal mechanisms that regulate and switch off neutrophil influx, 2) inappropriate or uncontrolled neutrophil activation within the lung, 3) inhibition of neutrophil apoptosis, and 4) impairment or saturation of the normal macrophage-dependent process for the removal of apoptotic neutrophils. Current *in vitro* data indicate that many factors operating at the inflamed site (*e.g.* cytokines, growth factors, chemotactic peptides, hypoxia, acidosis, *etc.*) serve a dual function in both priming and activating these cells, and delay apoptosis. The observation that the rate of eosinophil apoptosis can be accelerated by corticosteroid therapy *in vivo* suggests a novel mode of action for this drug and indicates that targeting this process in other granulocyte-dependent inflammatory conditions may offer a novel therapeutic approach in inflammatory lung disease.

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Keywords: Acute respiratory distress syndrome, apoptosis, lung inflammation, neutrophils.

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Neutrophils are major effector cells of the immune system and play a central role in the pathogenesis of many inflammatory lung disorders. Hence, the accumulation and activation of these cells at an inflamed site, while important for host defence against invading micro-organisms, is also responsible for releasing into the lung highly toxic proteases and reactive oxygen radicals. This "unwanted" consequence of neutrophil activation has been implicated in causing the lung parenchymal abnormalities associated with acute respiratory distress syndrome (ARDS), the airway wall damage observed in acute severe asthma and the inflammatory damage seen in many chronic interstitial lung diseases [1–3]. It has long been recognized, however, that, in the setting of acute bacterial pneumonia, neutrophil influx can occur on such a scale as to completely fill and obliterate the normal alveolar airspace and yet this process can resolve rapidly with minimal or no residual tissue damage. This simple paradox implies that the presence of neutrophils *per se* at an inflamed site does not automatically equate with tissue injury, even within an organ as delicate as the lung, and that other factors dictate whether damage ensues or is avoided. To date, most research has focused on trying to explain such differences in terms of the duration of neutrophil influx into the lung, virulence issues relating to the infecting micro-organisms and the presence or absence of other costimulatory cells. However, the recognition that neutrophils, when aged *in vitro*, undergo rapid and spontaneous apoptosis or "programmed cell death" has suggested a novel and quite alternative explanation for such differences *i.e.* that the timely death and active removal of extravasated neutrophils may be a key issue determining whether granulocytic inflammation resolves ("beneficial inflammation") or persists ("detrimental inflammation").

Neutrophil apoptosis and the resolution of inflammation

The process of apoptosis (fig. 1) is important for two main reasons: first, it switches off the secretory capacity of the neutrophil [4] and, secondly, it results in the recognition and prompt removal of these cells by inflammatory macrophages [5]. Since the plasma membrane of the neutrophil remains intact following apoptosis and the phagocytic engulfment of such cells fails to incite macrophage activation, this event appears to represent an "inbuilt", efficient and safe mechanism for the removal of potentially hazardous cells and may explain how it is that neutrophil inflammation can so frequently resolve without causing tissue damage. Likewise, if the process of apoptosis is delayed or perverted, the potential for these cells to degranulate and/or release other more damaging histotoxic agents increases dramatically. The whole concept that cell death may be beneficial has revolutionized our understanding of the events that control

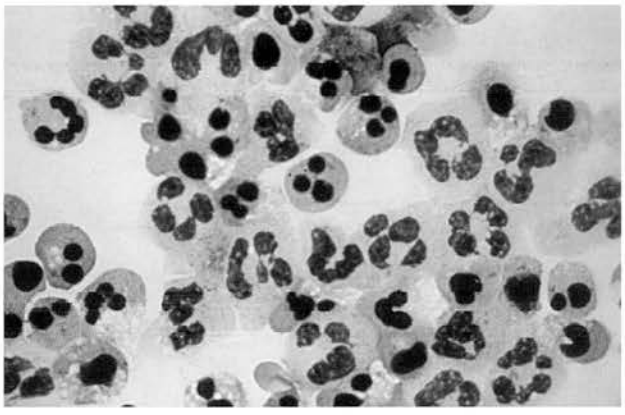

Fig. 1. – Morphological change associated with neutrophil apoptosis. Human neutrophils were purified from nuclear venous blood of healthy donors and cultured for 20 h in the presence of 10% autologous serum. Cytocentrifuge preparations were then performed revealing the clear morphological features of the apoptotic (denser nuclear staining) and non apoptotic (normal) neutrophils. Identical preparations using freshly isolated neutrophils demonstrate no evidence of apoptosis.

neutrophil behaviour at the inflamed site and offers many exciting and novel opportunities for developing anti-inflammatory drugs which may target this process. Although there are a number of recent reviews detailing the biology of neutrophil apoptosis [6], the principal aim of this article is to address how this event relates to inflammation in the lung.

Detection of neutrophil apoptosis in inflammatory lung disease

Recent data suggest that bacterial infection in the lung triggers a rapid but surprisingly transient efflux of neutrophils from the blood into the alveolar wall and airspace [2, 7] and that, unlike in the systemic circulation, these cells exit at the the pulmonary capillary level rather than from postcapillary venules [8]. For example, in experimental pneumonia caused by *Streptococcus pneumoniae*, neutrophil efflux only occurs during the first 6 h following instillation of the organism, and ^{111}indium-labelled autologous neutrophils fail to accumulate in the lung in patients with bacterial pneumonia, even when studied within hours of presentation (see [2]). Although granulocyte influx undoubtedly occurs on a more sustained basis in conditions such as chronic bronchial sepsis [7], the above data predict that in the setting of acute inflammation, neutrophil influx is switched off early and that events influencing neutrophil survival within tissues are likely to dominate the subsequent clinical course.

Neutrophil apoptosis has now been observed in a number of human diseases and experimental models including neonatal lung injury [9], ARDS [10], and experimental pneumonia and lung injury in animals [11], and supports previous predictions made on the basis of *in vitro* work [2]. The animal studies have also demonstrated a close correlation between the onset of neutrophil apoptosis in the lung, the subsequent ingestion of apoptotic cells by alveolar and inflammatory macrophages, and the resolution of the alveolitis [11]. A similar correlation has been made for the eosinophil; apoptosis and the subsequent clearance of these cells from the airways has been demonstrated in patients with acute severe asthma treated with systemic corticosteroids [12]. In this latter study, clinical improvement was mirrored by the appearance of apoptotic eosinophils in the sputum. Hence, there is little doubt that granulocyte apoptosis occurs *in vivo* and is particularly prominent in situations in which spontaneous or drug-induced resolution of inflammation occurs. Likewise, mice that lack the major β_2 integrin cell surface adhesion molecule CD11b/CD18 (which is required for phagocytosis-induced apoptosis) are unable to effectively clear neutrophils from the inflamed peritoneal cavity [13].

Functional consequences of neutrophil apoptosis

Time-lapse video microscopy studies indicate that neutrophil apoptosis is an energetic and rapidly executed event. The major morphological changes that occur include cell shrinkage, nuclear condensation and cytoplasmic vacuolation. These events are associated with the shedding of a number of cell surface receptors, including CD16 (FcγRIII) and the tumour necrosis factor receptor (TNFR)-1, and the externalization of phosphatidylserine, which is normally located only on the inner leaflet of the plasma membrane. This dramatic change in the morphology of the neutrophil is associated with a "functional isolation" of the cell, and an abrupt reduction in the cell's capacity to respond to stimuli that normally induce chemotaxis, degranulation or superoxide anion generation [4].

The second major consequence of neutrophil apoptosis is the acquisition of a phenotype that allows these cells to be recognized and engulfed by macrophages [5]. Although alveolar macrophages and fibroblasts all display some ability to phagocytose apoptotic granulocytes [14], this capacity appears dwarfed by the ability of "professional" inflammatory macrophages to engulf and degrade these cells, and this "eating" capacity can be further enhanced by a range of inflammatory mediators including granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ, interleukin-(IL)-1β and transforming growth factor-β1 [15]. Although the "ligand" on the apoptotic neutrophil cell surface that allows the senescent neutrophil to be recognized by macrophages is not known, the macrophage side of this interaction involves both the $\alpha_v\beta_3$ vitronectin receptor integrin and CD36, which bind thrombospondin, which engages the apoptotic neutrophil cell surface [5].

Macrophage uptake of apoptotic neutrophils has been observed at a number of inflammatory sites, including nonsuppurative arthritis, acute lung injury in neonates, ARDS, streptococcal lobar pneumonia, experimental peritonitis and resolving glomerulonephritis, and is thought to be the major disposal route of these cells *in vivo*. The crucial benefit of such a mechanism is that the phagocytic process removes cells that are potentially highly histotoxic in a "silent" and nonphlogistic manner [16]. Under *in vitro* conditions, blocking macrophage uptake using agents such as colchicine allows apoptotic cells to undergo secondary necrosis, causing the release of their granule contents, again underlining the need for neutrophil apoptosis to be co-ordinately linked to macrophage uptake [5].

Regulation of neutrophil apoptosis

The speed at which neutrophils undergo apoptosis when aged *in vitro* suggests the existence of a very strong and inbuilt apoptotic programme in these cells, which if triggered *in vivo*, could (and in streptococcal lobar pneumonia probably does) result in a very rapid and effective process for switching off and removing these cells from an inflamed site. Rather surprisingly therefore, it was revealed that the vast majority of input signals to the neutrophil (*e.g.* GM-CSF, lipopolysaccharide, complement factor 5a and (C5a), IL-6 and IL-8; fig. 2) cause a profound inhibition of apoptosis [17–19] and, moreover, that many of the pharmacological manipulations shown to induce apoptosis in other cell types (including the thymocyte and the lymphocyte) have the opposite effect on the apoptotic programme in the neutrophil. The most striking example of this difference is the effect of raising intracellular calcium or cyclic adenasine monophosphate, since both stimulate thymocyte apoptosis but delay this process in neutrophils [20, 21]. This early indication that neutrophils differ from most other inflammatory cells in their apoptotic programming was further supported by the observation that corticosteroids markedly delay apoptosis in the neutrophil yet clearly stimulate this process in the eosinophil [22]. This latter observation may explain, at least in part, the relative inability of corticosteroids to influence the clinical outcome of many neutrophil-dominated inflammatory diseases such as ARDS.

It has also been demonstrated that the rate of neutrophil apoptosis can be severely inhibited by hypoxia [23]. This influence is again contrary to the effect of hypoxia on apoptosis in other cells types, *e.g.* neurons and gut-derived epithelial tumour cells, and suggests that hypoxia may be a further factor operating to delay the resolution of neutrophil-mediated tissue inflammation.

In view of these data, recent studies have focused on how neutrophil apoptosis may be stimulated or, at the very least, how the above largely cytokine-mediated survival effects may be blocked. This has proved a somewhat frustrating challenge since although agents such as TNF-α and Fas ligand (Fas-L) are both capable of inducing neutrophil apoptosis through binding to the cell membrane p55 and p75 TNF-α receptors and Fas respectively [24–27], the magnitude of the killing effect, at least *in vitro*, is small. Furthermore, neutrophils previously primed by agents such as platelet-activating factor, IL-8 or lipopolysaccharide, or cells isolated from an inflammatory focus, appear unresponsive to the same signals [26, 28, 29]. This implies that the early and often intense increase in TNF-α levels observed in many acute inflammatory lung states may be unable to promote effective neutrophil removal if the cells within the lung have been costimulated by other priming or activating agents. Early reports suggested that neutrophils actively produce and release Fas-L and thereby regulate their own survival *via* autocrine or paracrine Fas-L/Fas interactions [27, 30]. However, Fas-L/Fas-mediated apoptosis may not be as important in regulating neutrophil survival *in vivo* since inflammatory neutrophils harvested from Fas-L (*gld*) and Fas (*lpr*)-deficient mice undergo apoptosis at an identical rate to cells from normal mice [31].

Other physiological stimuli known to enhance the rate of neutrophil apoptosis include human immunodeficiency virus infection [32], ingestion of *Escherichia coli* [33] and other particulate material [13], and, interestingly, migration of cells through an endothelial monolayer [34]. This latter observation may explain why apoptotic neutrophils are rarely if ever observed in circulating blood, and may relate to the previously noted ability of β_2 integrins to facilitate constitutive and TNF-α-stimulated neutrophil apoptosis [13, 34]. There is also an early and fascinating suggestion that bronchoalveolar lavage fluid recovered from animals with experimental streptococcal (resolving) pneumonia contains a factor that drives neutrophil apoptosis *in vitro* and that this factor(s) is absent in *E. coli* (non-resolving) pneumonia [35].

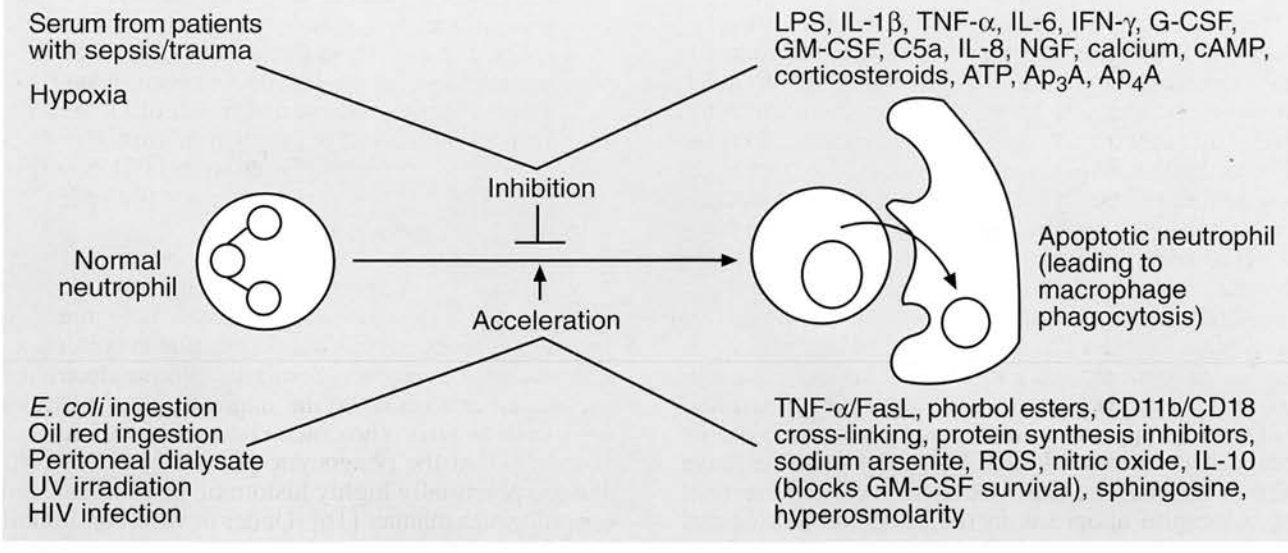

Fig. 2. – Regulation of neutrophil apoptosis. Summary of the physiological (left) and pharmacological (right) manipulations demonstrated to either inhibit or accelerate the rate of neutrophil apoptosis *in vitro*. *E. coli*: *Escherichia coli*; UV: ultraviolet; HIV: human immunodeficiency virus; LPS: lipopolysaccharide; IL: interleukin; TNF-α: tumour necrosis factor-α; IFN-γ: interferon-γ; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; C5a: complement factor 5a; NGF: nerve growth factor; cAMP: cyclic adenosine monophosphate; ATP: adenosine triphosphate; Ap_3A, Ap_4A: diadenosine polyphosphates; FasL: Fas ligand; ROS: reactive oxygen species.

Although the intracellular pathways mediating the above effects are uncertain, the proapoptotic effect of TNF-α, unlike its priming/activation functions, are uniquely dependent on ligation of TNFR75 in addition to TNFR55, and both TNF-α and Fas-L-induced killing are blocked by compounds that inhibit the caspase family of cysteinyl proteases, which orchestrate cell death in other mammalian cells. The major nuclear and cytosolic targets of caspases in the neutrophil are, again, uncertain but appear to include gelsolin, which acts to sever actin filaments and induce cells to round up, detach and undergo nuclear fragmentation [36], and a Ca^{2+}/Mg^{2+}-independent acid endonuclease [37]. The ability of protein synthesis inhibitors to induce neutrophil apoptosis also implies that these normally short-lived cells are "preprogrammed" to die and that an actively produced antiapoptotic protein factor or factors exist to prevent premature cell death. Intriguingly, mature neutrophils do not possess Bcl_2, a major antiapoptotic factor present in most other cells [29, 38], and it has been proposed that the loss of this protein, which occurs during the final stages of granulocyte maturation, removes an important brake on the apoptotic capacity of the neutrophil prior to entry into the circulation. Current contenders for such survival proteins include other members of the Bcl_2 family, namely Mcl-1 and Bax [39].

Potential for therapeutic manipulation

From the above line of argument, it follows that devising a therapeutic strategy based on driving programmed cell death or blocking the antiapoptotic effects of certain agents should, if executed at the appropriate stage of the inflammatory response, offer a novel and effective form of anti-inflammatory therapy. "Proof principle" for this is the well-recognized and major proapoptotic effect of glucocorticosteroids in eosinophils and the ability of Fas-L administration to induce resolution of eosinophilic inflammation in the airways [40]. Such selective targeting is not yet available for the neutrophil, but there are experimental data to support both of the above approaches. First, although the "solo" killing effect of TNF-α in neutrophils appear to be modest and are easily overcome by other costimuli, it is now possible to increase dramatically the efficacy of this response by coincubating neutrophils with the nuclear factor-κB inhibitor gliotoxin [41], which also unmasks a capacity for TNF-α to drive eosinophil apoptosis. Likewise, the protein synthesis inhibitor cycloheximide, used at a concentration that alone does not influence the rate of constitutive apoptosis, can also enhance TNF-α-mediated neutrophil apoptosis [41]. Secondly, the anti-inflammatory cytokine IL-10 has been shown to be able to block the survival (anti-apoptotic) effect of lipopolysaccharide *in vitro* [42], and a similar effect has been demonstrated with certain synthetic cell-permeable inhibitors that block certain intracellular second messenger phosphorylation pathways [43]. The very unusual and atypical apoptotic programme that exists in the neutrophil, and the ability to deliver drugs in a selective manner to the lungs, fuels current optimism that such an approach may be feasible.

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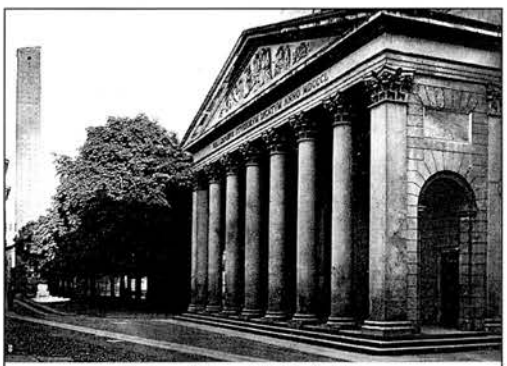

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Kinetics of equine neutrophil elastase release and superoxide anion generation following secretagogue activation: a potential mechanism for antiproteinase inactivation

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(mean ± SEM, $n = 11$ individual horses) per neutrophil, respectively. This represents twice as much elastase as previously found in the equine neutrophil and a comparable amount to that reported in human neutrophils. Immunolocalisation demonstrated that ENE 2A has a granular distribution within the cytosol of neutrophils, whereas API exhibits a uniform non-granular cytoplasmic appearance. In addition the kinetics of simultaneous generation and release of superoxide anions (SOA) and release of ENE 2A from equine neutrophils, stimulated in vitro by zymosan-activated serum (ZAS) in the presence and absence of the cation chelator ethylene glycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), showed a close relationship between total SOA generation and total ENE 2A release during the initial 90 min post-ZAS stimulation and the dependence of both events on extracellular cations. In conclusion these studies have shown that horse and human neutrophil elastase content and mediator release functions are more closely matched than was previously thought. This suggests that the species differences in pathology resulting from neutrophil-mediated respiratory disease are determined by other factors such as differences in the abundance and function of intra- and extra-cellular protease inhibitors.

Keywords: Horse; Equine; Neutrophil; Elastase; Alpha-1-proteinase inhibitor

1. Introduction

Neutrophil elastase (NE) is a major component of the neutrophil's proteolytic armoury being essential for migration to extravascular compartments (Henson and Johnston, 1987), oxygen independent bactericidal activity (Ganz et al., 1987) and the remodelling of damaged tissues. However, inappropriate or excessive release of NE can be detrimental to host tissues as NE is a powerful serine proteinase (Bieth, 1986) capable of degrading many structural proteins including virtually any component of the pulmonary extracellular matrix (Dale et al., 1972; Ganz et al., 1988; Haslett et al., 1989; Bokoch, 1995; Downey et al., 1995). Consequently, delicate alveolar structures of the lung are extremely sensitive to unregulated NE activity. Neutrophil elastase is synthesised in bone marrow precursor cells, especially promyelocytes (Fouret et al., 1989), and found in the primary cytoplasmic granules of the mature circulating cell (Cramer et al., 1989). Under normal conditions the activities of NE are precisely controlled by serine proteinase inhibitors (serpins) such as alpha-1-proteinase inhibitor (API) (Gadek et al., 1981a; duBois et al., 1991; Mason et al., 1991), secretory leukocyte proteinase inhibitor (SLPI) (Sallenave et al., 1997) and alpha-2-macroglobulin (Travis and Salvesen, 1983). Plasma derived API is the main regulator of NE activity in the lower respiratory tract (Gadek et al., 1981b). Its critical importance is highlighted by a human hereditary disorder associated with API deficiency and a greatly reduced plasma concentration of API which leads to the development of panacinar pulmonary emphysema in the third to fourth decade of life (Laurell and Eriksson, 1963; Hayes et al., 1975; Janoff et al., 1977; Perlmutter, 1996).

The recognition of pulmonary emphysema in some API sufficient humans, as well as those with API deficiency, suggests that NE host tissue damage results from two basic mechanisms; either a fault in the antiproteinase screen (e.g. reduced amount or inactivation), or failure due to an excessive burden of proteolytic enzymes which

overwhelm the capacity of the inhibitors present. These are two extremes and it is probable that a combination of the two occur in many cases of pulmonary emphysema in API sufficient individuals giving rise to 'the proteinase : antiproteinase theory of lung disease' (Shapiro, 1995). Human API is susceptible to inactivation by a host of different oxidants produced by activated neutrophils (Ossanna et al., 1986; Ottonello et al., 1992; Ras et al., 1992), macrophages (Hubbard et al., 1987; Mast et al., 1991) and cigarette smoke (Travis et al., 1980). This susceptibility to oxidative inactivation is due to the presence of a methionine residue in the P_1 position on the exposed reactive site loop (Johnson and Travis, 1979; Padrines et al., 1989) which, when oxidised to methionine sulphoxide, results in a thousand fold reduction of the association rate constant (k_{ass}) of API with NE (Matheson et al., 1982). This phenomenon has also been reported for equine API (Patterson and Bell, 1989). Inactivation of API by SOA generation may allow neutrophils to induce a micro-environment around the cell in which NE activity can proceed unimpaired (Ossanna et al., 1986; Ras et al., 1992). For this theory to be valid a rapid respiratory burst resulting in extra-cellular SOA must be temporally co-ordinated with the release of NE.

Equine chronic obstructive pulmonary disease (COPD) is considered to be an immune complex-mediated hypersensitivity response to inhaled antigens characterised by airway obstruction and neutrophil recruitment into the airways (Robinson et al., 1996). Histopathology shows accumulation of neutrophilic exudate down to the bronchiolar level of airways, however, peribronchiolar emphysema is minimal and the deeper alveoli are rarely affected (Kaup et al., 1990). Panacinar and centrilobular emphysema, prominent features of some neutrophil associated pulmonary diseases in humans (Creagh and Krausz, 1992), are not documented in equine COPD (Kaup et al., 1990) suggesting a major species difference, either in the susceptibility of the alveoli to NE or the amount, or activity, of the NE present. Previous reports suggest that equine neutrophils contain a lower concentration of elastase (0.4 pg/cell measured by elastinolytic activity of cell extracts, Dubin and Koj, 1986) compared to human neutrophils (1.1 pg/cell measured by competitive ELISA of cell extracts, Tetley et al., 1989). If these figures are correct the explanation for the different patterns of emphysema present in the two species may simply be due to the lower concentration of elastase available in the horse which is therefore more easily controlled by inhibitors present in the locality. However, as NE inhibitors have been described in the cytosol and NE containing granules of both human (duBois et al., 1991; Mason et al., 1991; Sallenave et al., 1997) and horse neutrophils (Dubin, 1977; Potempa et al., 1988; Korzus et al., 1991; Dubin et al., 1992; Kordula et al., 1993), the measurement of elastinolytic activity may underestimate the amount of elastase present in equine neutrophils as an unknown proportion may be inhibited during the extraction process. However, it must be remembered that tissue damage in vivo is caused by active elastase and that while extraction methods may result in apparent loss of elastase activity immunological techniques are also imperfect in that they do not indicate what proportion of the protease is functionally available. The aim of the study was to determine whether the content and intracellular compartmentalisation of NE and API and the kinetics of release of NE and SOA generation from equine neutrophils, after exposure to secretagogue, were comparable with the previously published data for human neutrophils. To enable a more accurate determination of ENE in neutrophils using

immunological techniques which would allow direct comparison with the human literature antibodies specific for ENE 2A (the most abundant NE found in equine neutrophils, Von Fellenberg et al., 1985; Dubin and Koj, 1986) were produced.

Using immunological techniques, (i) the presence and distribution patterns of both ENE 2A (free or complexed) and equine API in venous blood neutrophils was demonstrated, (ii) the amount of ENE 2A per cell was shown to be greater than previously reported and (iii) the release kinetics of ENE 2A and generation of SOA were compared suggesting the potential for synergistic interaction of these mediators during the period of the oxidative burst.

2. Materials and methods

2.1. Reagents and chemicals

Sodium citrate 3.8% was purchased from Phoenix Pharmaceuticals, (Gloucester, UK), Percoll and CNBr-activated Sepharose from Pharmacia (Uppsala, Sweden), Diff Quik™ from B. M. Brown (Reading, Berks, UK) and dried skimmed milk from J. S. Sainsbury (London, UK). FluoroLink-Ab™ Cy3™ Labelling Kit was purchased from Amersham Life Science (Arlington Heights, IL, USA). Vectashield, 3,3′-diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC) from Vector Laboratories (Peterborough, UK) and microtitre plates and 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate from Dynex Technologies (Billingshurst, West Sussex, UK). Crystal/mount™ was purchased from Biomeda (Foster City, CA, USA). All other reagents were of tissue culture or reagent grade and supplied by Sigma Chemicals (Poole, Dorset, UK).

Zymosan-activated serum (ZAS) was used as a biological source of the complement fragment C5a in order to obtain a species homologous, receptor mediated, physiological stimulus for equine neutrophils. Serum was prepared by the recalcification of platelet rich plasma followed by incubation for 90 min at 37°C in an humidified 5% CO_2 atmosphere. Zymosan (5 mg/ml) was added to the serum followed by sonication, incubation at 37°C for 60 min and centrifugation at 1400 × *g* for 15 min. Heat inactivated zymosan-activated serum (HIZAS) was produced by an identical procedure except that the serum was heat inactivated at 56°C for 30 min before addition of zymosan. Both supernatants were stored in aliquots at −20°C.

2.2. Neutrophil isolation

Venous blood neutrophils were isolated by modification of a previously published method (Haslett et al., 1985). All blood was taken by jugular venipuncture and anticoagulated with 1 ml of 3.8% sodium citrate per 10 ml of blood. Free sedimentation under gravity for 30 min at room temperature revealed an upper leukocyte rich plasma layer which was aspirated and centrifuged at 380 × *g* for 6 min. Platelet-rich plasma supernatant was decanted and centrifuged at 2400 × *g* for 20 min to prepare platelet poor plasma (PPP). The initial leukocyte rich pellet, obtained typically from 80 ml of whole blood, was resuspended in 2 ml PPP in a 15 ml polystyrene tube and underlayered with

40% and 52% PPP/Percoll solutions prior to centrifugation at 255 × *g* for 12 min. Two tight bands appeared, cytospins preparations from these revealed the mononuclear cells were held up in the PPP/40% Percoll interface and neutrophils in the 52%/40% interface. Neutrophils harvested from this layer were washed sequentially in PPP, three times in Dulbecco's PBS without calcium and magnesium (w/o)/0.1% BSA and once in Dulbecco's PBS with calcium and magnesium/0.1% BSA prior to resuspension in the latter. All procedures were performed at room temperature. Purity was assessed by differential count of Diff-Quik™ stained cytospin preparations and viability by trypan blue exclusion.

2.3. Measurement of equine API by ELISA

Purification of equine API, production and characterisation of anti-equine API antibodies used in this study and the sandwich ELISA for API have previously been reported by this group (Scudamore et al., 1994; Dagleish et al., 1998). Briefly ELISA plates were coated with purified sheep anti-equine API (1 mg/ml). Standards (in triplicate) and samples (in duplicate) were then added to the wells and bound API was detected by addition of the same antibody conjugated to HRPO and the peroxidase substrate TMB. The optical density of the colour reaction in the wells was measured at 450 nm on a MR7000 ELISA plate reader (Dynex Technologies, Billingshurst, West Sussex).

2.4. Purification of equine neutrophil elastase 2A and production of a sheep anti-ENE 2A antibody

ENE 2A was purified from 6 l of whole blood collected immediately post-mortem. Neutrophil granules were separated by cell homogenisation followed by centrifugation and solubilised by stirring overnight in 1 M NaCl, 0.05% Triton-X 100 at 4°C. ENE 2A was purified from the resultant supernatant using ion exchange chromatography and stored at −70°C following buffer exchange in to PBS (Scudamore et al., 1993).

Antisera to ENE 2A was raised by injecting a sheep with a complex of API : ENE 2A prepared by pre-incubation of 50 μg of ENE 2A and 100 μg of API at room temperature for 30 min. Three similar injections were given over a 6 month period in either Freund's complete (initial challenge) or incomplete adjuvants (subsequent challenges). Antibodies were purified from sera by affinity chromatography using a column of purified ENE 2A linked to CNBr-activated Sepharose 4B. Purified antibody was stored in PBS in aliquots at −20°C.

2.5. SDS-PAGE and Western blotting techniques to characterise the proteolytic activity and demonstrate immunodetection of free and complexed ENE 2A and API

Crude neutrophil extracts were prepared by five cycles of freeze/thaw extraction of pellets of 126×10^6 purified equine neutrophils in 600 μl reducing on non-reducing sample buffer containing 1 M NaCl. The extract in reducing buffer was heated to 95°C and then both extracts were centrifuged (20 000 × *g*, 30 min at 4°C) and the supernatants

retained and stored at −70°C. Four identical immunoblots were prepared by separation of purified ENE 2A, neutrophil extract, API and API : ENE 2A complex (0.5 μg, 20 μl of the above extracts, 1.0 and 1.5 μg per lane, respectively) by 10% SDS-PAGE under reducing and non-reducing conditions, the proteins being transferred to Immobilon-P by semi-dry Western blotting. Non-specific binding to the blots was blocked by 3% dried skimmed milk in PBS (3% MPBS) prior to addition of sheep anti-ENE 2A Ab at 5 μg/ml (in 3% MPBS), or 0.5% Tween 80 in PBS (PBST) prior to addition of monoclonal anti-equine API (mAb 1B, Dagleish et al., 1998), at 40 μg/ml in PBST. Donkey anti-sheep IgG: HRPO conjugate at 1/4000 dilution in 3% MPBS, or goat anti-mouse IgG (FAB-specific): HRPO diluted 1/5000 in PBST plus a DAB peroxidase substrate kit were used to visualise binding of the respective primary antibodies.

Proteolytic activity of purified ENE 2A, neutrophil extract, API and API : ENE 2A complex was assessed by incorporating 20 mg azocasein per 10 ml of resolving gel solution in 10% SDS-PAGE under non-reducing conditions (Horie et al., 1984). Sample loading was as above. Following electrophoresis the gel was washed three times in 2.5% Triton-X 100 for 30 min before overnight incubation in PBS. The resultant gel was stained with Coomassie brilliant blue and destained in the usual manner; proteolysis being seen as a clear band on the gel.

2.6. *Immunofluoresent detection of ENE 2A and API in equine leukocytes*

Citrated whole equine blood (10 ml) was allowed to stand for 30 min to allow sedimentation of erythrocytes. Leukocyte rich plasma was removed and centrifuged (330 × *g*, 6 min) at 21°C, the resultant cells were washed and centrifuged (235 × *g*, 6 min, 21°C) four times in Dulbecco's PBS without calcium and magnesium (w/o). Cells were resuspended at 0.5×10^6/ml in Dulbecco's PBS with calcium and magnesium, 100 μl samples were used to make cytospin preparations (300 rpm, 3 min, Cytospin 3, Shandon, Pittsburgh, USA) onto TESPA (3-aminopropyltriethoxysilane) coated slides which were fixed in 4% paraformaldehyde in PBS (45 min at 45°C) and stored in 70% ethanol at 4°C.

Sheep anti-ENE 2A and sheep anti-API (Dagleish et al., 1998) antibodies were conjugated, using kits, with Cy3™ and FITC, respectively. Endogenous fluorescence was quenched with DAB (0.4 mg/ml PBS), 5% normal sheep serum (5% NSS) blocked non-specific binding followed by application of anti-ENE 2A : Cy3 at 20 μg/ml and anti-API : FITC at 14 μg/ml, both in 5% NSS (40 μl of each/slide). Negative controls were treated identically except that the primary antibodies were substituted by normal sheep IgG conjugated to Cy3 and FITC. After washing, cytospin preparations were mounted in Vectashield.

2.7. *ELISA for equine neutrophil elastase 2A*

ELISA plate wells were coated with 50 μl of affinity purified sheep anti-ENE 2A (1.5 μg/ml) in 0.1 M carbonate/bicarbonate buffer (pH 9.6). After incubation at 4°C overnight, plates were washed six times with 0.9% NaCl containing 0.05% Tween 20 (0.9% NaCl-T20). Samples (in duplicate) and standards (in triplicate) were diluted in 5%

MPBS containing 0.05% Tween 20 (5% MPBST20), and 50 μl added per well. Purified ENE 2A was used as a standard over the range 0.5–4.0 ng/ml. Wells containing either 50 μl 5% MPBST20 or samples from a single stored batch of ENE 2A constituted negative and positive controls, respectively. Plates were incubated at room temperature for 1 h followed by washing six times in 0.9% NaCl-T20. 50 μl of affinity purified sheep anti-ENE 2A conjugated with HRPO (1 μg/ml in 5% MPBST20) was added per well and plates incubated a for a further 1 h at room temperature followed by a final six washes in 0.9% NaCl-T20. To initiate colour development 50 μl of TMB was added per well and the reaction terminated by addition of 25 μl of 0.18 M H_2SO_4. The optical density was measured at 450 nm on a MR7000 ELISA plate reader (Dynex Technologies, Billingshurst, West Sussex). The ELISA was used to compare the detection of ENE 2A in the free and complexed state by addition of samples of purified ENE 2A pre-incubated in the presence or absence of excess purified API (samples containing ENE 2A in the range 0.5–4 ng/ml were examined). The amount of ENE 2A present in equine neutrophils was measured in samples taken from the venous blood of 11 horses (six mares, four geldings and one stallion, median age 10 years, range 5–19 years). Following purification as previously described neutrophils were resuspended at 5×10^6 cells/ml in Iscove's modified Dulbecco's PBS freeze/thawed five times, centrifuged (13 800 × *g*, 10 min at 4°C) and the resultant supernatant assayed for both ENE 2A and API content.

2.8. Zymosan–activated serum stimulation of superoxide generation and elastase release from equine neutrophils

A sample of the purified neutrophils ($n = 6$ individual horses, 2 geldings, 4 mares, mean age 15.8 years, range 8–29 years) was resuspended at 12.5×10^6 cells/ml in Dulbecco's PBS/0.1% BSA at 37°C. Basal (PBS), ZAS, HIZAS and ZAS with EGTA (final concentration of EGTA 4 mM) stimulated superoxide generation was measured by lucigenin-dependent chemiluminescence (LDCL) (Allen, 1986; Benbarek et al., 1996) using a ML 3000 microtitre plate luminometer (Dynex Technologies, Billingshurst, West Sussex, UK). Lucigenin (0.25 mM in Dulbecco's PBS/0.1% BSA at 37°C) 100 μl and 1×10^6 freshly isolated cells (80 μl) were added to each well. Dose response curves were derived for SOA generation and ENE 2A release in response to ZAS stimulation to determine the optimal amount of secretagogue (data not shown). After a 5 min equilibration phase, basal LDCL was recorded over 12 min to ensure that cells were not basally activated (stable basal peak < 0.005 RLU), followed by addition of 20 μl of buffer (PBS), ZAS, HIZAS or ZAS with EGTA to wells (final cell concentration 5×10^6/ml). Chemiluminescence was recorded to determine mean peak and integral (area under the curve) chemiluminescence values from triplicate wells over a 90 min period (area under the curve, determined by GraphPad Prism™ version 2.0, GraphPad Software, San Diego, CA, USA).

Concurrently the rest of the purified neutrophils were resuspended at 5.56×10^6 cells/ml in Dulbecco's PBS/0.1% BSA and 4.5 ml aliquots placed into universal containers. One-half millilitre of either PBS, ZAS, HIZAS or ZAS with EGTA was added (final cell concentration 5×10^6/ml), following gentle mixing 0.5 ml samples were then withdrawn at 0 (immediately after addition of secretagogues), 15, 30, 45, 60, 90, 120 and 150 min.

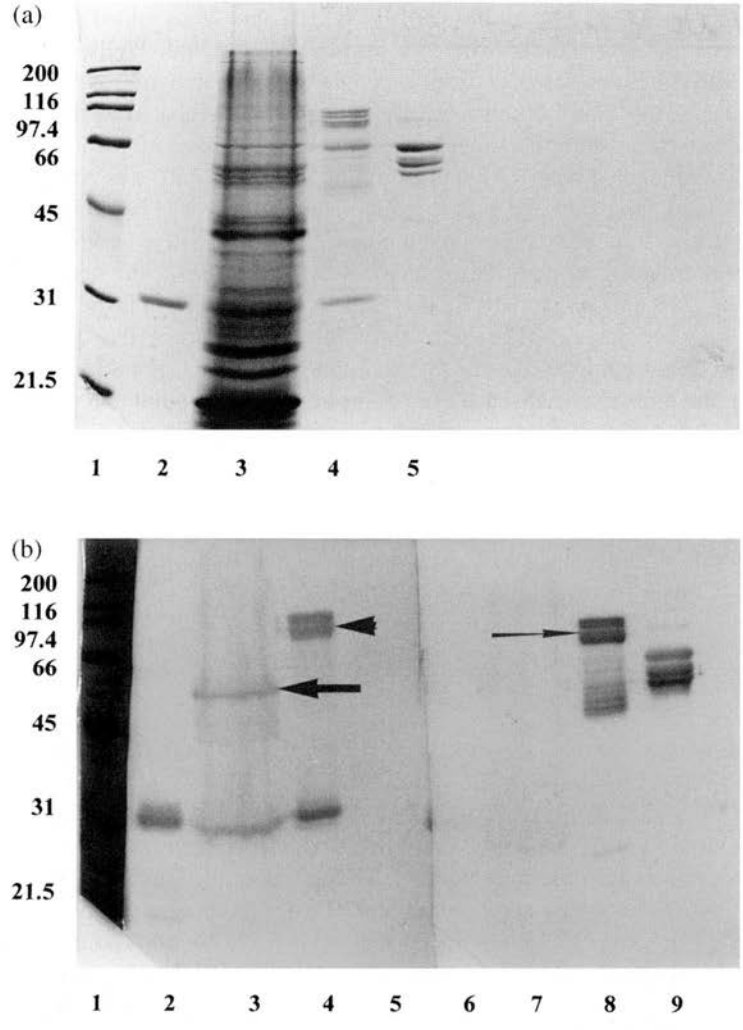

Fig. 1. Characterisation of sheep anti-ENE 2A antibody. (A) 10% SDS-PAGE of determining antigens under reducing conditions. Molecular weight markers (MW in kDa), purified equine neutrophil elastase 2A (ENE 2A) (0.5 μg/lane), neutrophil extract (20 μl/lane), API : ENE 2A complex (1 mg: 0.5 μg/lane) and purified API (1 μg/lane) in lanes 1–5, respectively. Note the presence of uncomplexed ENE 2A and API as well as complex in lane 4. (B) Analysis of antibody specificities. Western blot of gel under reducing conditions, similar loading to gel in Fig. 1A. Lane 1: Coomassie brilliant blue stained molecular weight markers, lanes 2–5 probed with anti-ENE 2A antibody, lanes 6–9 with anti-API antibody (mAb 1B). Lane contents 2, 3, 4 and 5 repeated in lanes 6, 7, 8 and 9, respectively. Note: (i) 31 kDa band common to lanes 2, 3 and 4 corresponding to ENE 2A, (ii) 55 kDa band in lane 3 (thick arrow) suggestive of ENE 2A : HLEI complex, (iii) API : ENE 2A complex in lanes 4 (arrow head) & 8 (thin arrow) (MW 79–108 kDa) showing both antibodies recognise ENE 2A : API complex, (iv) multiple isoforms of uncomplexed API in lane 8 below API : ENE 2A complex, (v) uncomplexed ENE 2A is not recognised by mAb 1B in lane 8 (vi) equine API multiple isoforms (MW 57–69 kDa) present in lane 9. Differences in molecular weight between API in lanes 8 and 9 are probably due to proteolytic cleavage of uncomplexed API in lane 8 by uncomplexed ENE 2A. (C) Proteolytic activity of purified antigens and

Each sample was centrifuged at 13800 × *g* for 10 min at 4°C, the supernatant decanted and stored at −70°C until analysis by ELISA.

2.9. Statistical analysis

Intra and inter-ELISA plate variations for ENE 2A evaluations were calculated from the values obtained for the positive control samples giving coefficients of variation of 1.3 and 16.1%, respectively. All analyses of superoxide anion generation and elastase release between neutrophil stimulants were by Kruskal-Wallis and Mann-Whitney Tests using a 95% confidence interval. Results were considered to be significant with $p < 0.05$.

3. Results

3.1. Affinity purified antibodies specifically recognise their complexed and uncomplexed respective proteins

Separation of purified ENE 2A by 10% SDS-PAGE under reducing conditions produced a single band of MW 31 kDa (Fig. 1A) in agreement with our previous study (Scudamore et al., 1993). Purified API isoforms and API : ENE 2A complex had MW from 57–69 kDa and 79–108 kDa, respectively (Fig. 1A), the ranges reflecting the differing MW of API isoforms (Patterson et al., 1991). Western blots probed with anti-

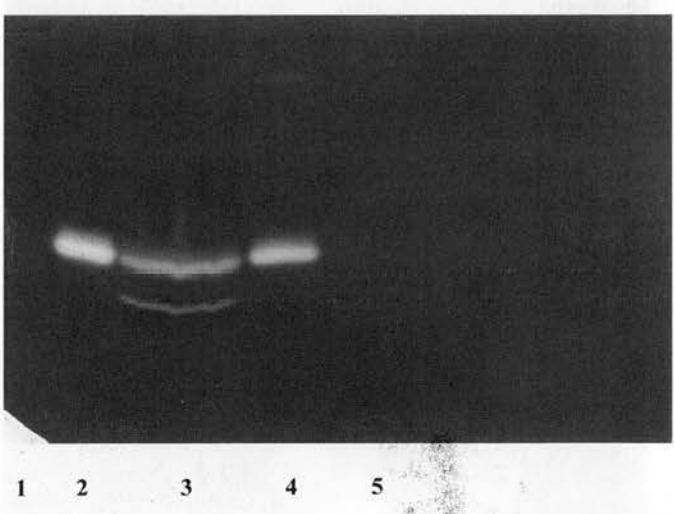

Fig. 1. (*Continued*)
neutrophil contents. 10% SDS PAGE under non reducing conditions incorporating 20 mg azocasein/10 ml of resolving gel. Loading is identical in concentration to gel 1A. Clear bands denote proteolysis. The clear band common to lanes 2, 3 and 4 represents ENE 2A. Note lack of any proteolysis above this common line in lane 3 yet two smaller bands below it. Also note slight proteolysis in the API : ENE 2A complex region of lane 4 depicting the lack of complete inhibition in this 'pseudo-irreversible' reaction.

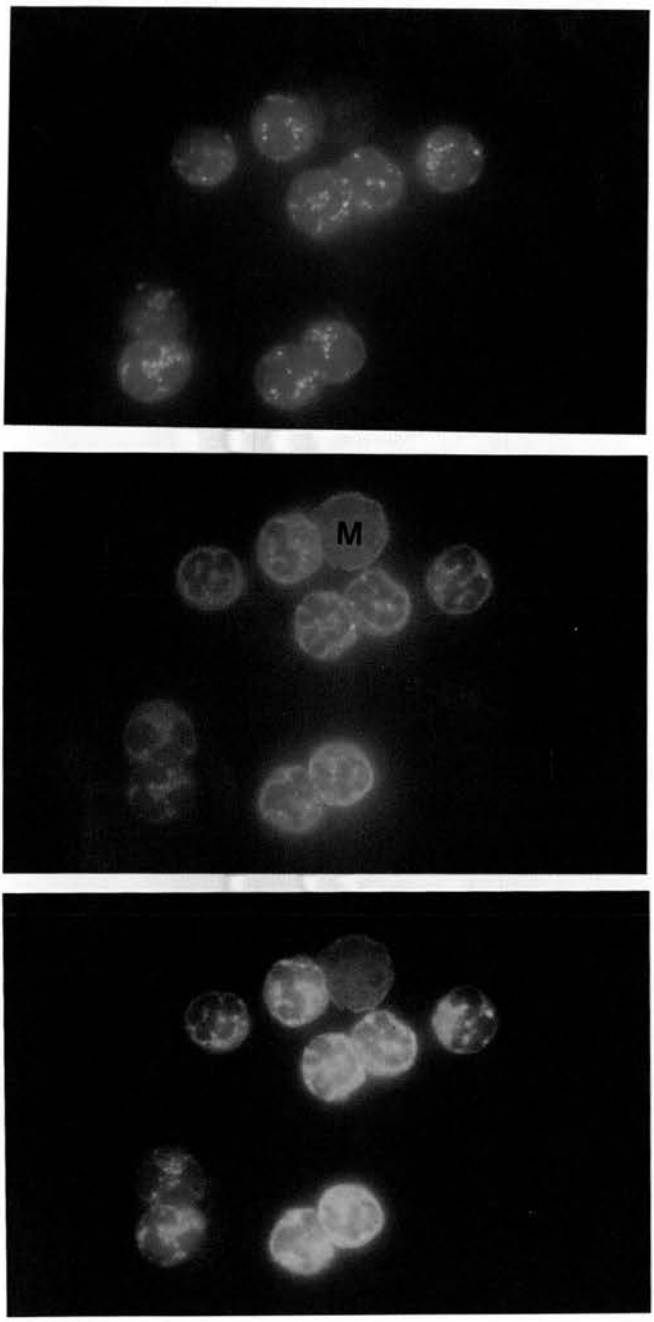

Fig. 2. Localisation of ENE 2A and API in venous blood leukocytes. Studies performed on cytospin preparations of mixed populations of equine venous blood leukocytes (100 µl of 0.5×10^6 cells/ml per cytospin). Original magnification of all figures × 1000. (A) Immunolocalisation of ENE 2A. Immunofluorescence using sheep

ENE 2A or anti-equine API antibodies showed no cross reactivity to API or ENE 2A, respectively, in reduced (Fig. 1B) or non-reduced states (data not shown), yet both clearly recognised API : ENE 2A complex. Further scrutiny of the specificity of the sheep anti-ENE 2A antibody showed that it identified both its target protein in the presence of soluble neutrophil contents and a band of 55 kDa MW (Fig. 1B). On the azocasein gel proteolysis was absent in the equivalent area of this higher MW band yet all uncomplexed ENE 2A displayed proteolytic activity (Fig. 1C). Two smaller bands of proteolysis of lower MW than uncomplexed ENE 2A were also detected demonstrating the presence of other proteases in the neutrophil extract. Purified API demonstrated no proteolytic activity but the API : ENE 2A complex exhibited a trace amount (Fig. 1C).

3.2. Equine neutrophils contain cytosolic API and neutrophil elastase with different patterns of distribution

Immunofluorescent labelling of a mixed population of venous blood leukocytes with sheep anti-ENE 2A antibody clearly demonstrated the presence of ENE 2A in multiple cytosolic granules in neutrophils (Fig. 2A). Other cell types were negative for ENE 2A as were all cell nuclei.

In contrast a more uniform, less intense and non-granular pattern of immunostaining was seen in the cytosol of neutrophils when probed with an anti-API antibody (Fig. 2B). Cytoplasmic staining was also present in some mononuclear leukocytes.

Double immunofluorescence emphasised the contrasting distribution patterns of API and ENE 2A in the cytosol of neutrophils but also indicated that the two proteins were partially colocalized (Fig. 2C). Negative control preparations were devoid of immunofluorescence.

3.3. Elastase and API content per neutrophil

The presence of intracellular ENE 2A inhibitors (Kordula et al., 1993, current study) capable of forming complexes with NE during the extraction process meant that it was important that the ELISA technique used recognised ENE 2A in both the free and complexed form. Using the ELISA reported in this study no significant difference in OD values was found between samples containing equivalent concentrations of ENE 2A in the presence or absence of an excess of API ($p = 0.07$). The ELISA techniques were then used to measure ENE 2A and API in supernatants of cell extracts of purified neutrophils

anti-equine neutrophil elastase 2A (ENE 2A) antibody labelled with Cy3™ (red) for visualisation. Note granular pattern of fluorescence confined to cytoplasm of neutrophils. (B) Immunolocalisation of Equine Alpha-1-Proteinase Inhibitor. Immunofluorescence of the same field of cells in 2A but using sheep anti-equine API labelled with FITC for visualisation (green). Positive fluorescence representing API is variable in intensity but remains non-granular in nature. A monocyte (M) also shows positive fluorescence for API. (C) Co-localisation of ENE 2A and API. Dual immunofluorescence of same field of cells as seen in A and B. This clearly illustrates the granular nature of ENE 2A and the non-granular nature of API. Note that API distribution covers the whole of the cells cytoplasm (green) but ENE 2A distribution is restricted to certain parts (yellow).

(94.2% neutrophils ± 0.9, cell viability of 99.8 ± 0.8%, mean ± SEM). Mean elastase content per neutrophil was 0.813 pg ± 0.179 SEM. Mean API content was 0.021 pg ± 0.003 SEM per neutrophil.

3.4. Zymosan-activated serum induces both superoxide anion generation and elastase release with complimentary kinetics

In view of the fact that in this study the ENE 2A content of neutrophils was found to be comparable to that reported in humans the possibility of a difference in the kinetics of ENE 2A release and SOA generation between the species was investigated.

The ZAS induced respiratory burst produced a peak SOA generation at 25 min post-stimulation, with complete desensitisation of the response by 90 min, despite the continued presence of the agonist (Fig. 3). Comparison of the total amount of SOA produced over 90 min, as represented by the areas under the curves, show that a statistically significant greater quantity of SOA was generated by neutrophils stimulated with ZAS when compared (Kruskal–Wallis test) to the amounts generated in response to the other three stimuli (ZAS versus HIZAS $p = 0.01$, ZAS vs. PBS $p = 0.005$ and ZAS versus ZAS + EGTA $p = 0.005$). Stimulation with HIZAS resulted in similar kinetics for SOA generation but with a significant reduction in magnitude (ZAS versus HIZAS $p = 0.01$). Cells stimulated with PBS or ZAS + EGTA released negligible amounts of SOA. Zymosan-activated serum stimulation in the presence of EGTA abolished the SOA generation such that the LDCL response was not significantly different to control cells treated with PBS ($p = 0.9$).

In contrast, the kinetics of ENE 2A release (in response to ZAS stimulation) were prolonged with continuing accumulation in supernatants for up to 150 min (the final sampling point, Fig. 4). ZAS stimulated neutrophils consistently released significantly greater amounts of ENE 2A over the whole 150 min (when analysed by Kruskal–Wallis test) compared with values for PBS ($p = 0.005$). However, the ENE 2A released after

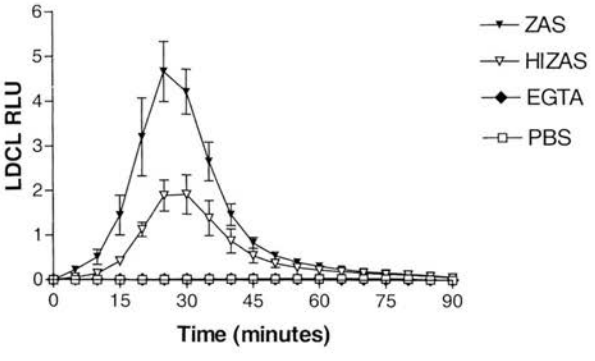

Fig. 3. Superoxide anion (SOA) generation from ZAS, heat inactivated ZAS (HIZAS), ZAS + ethylene glycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and phosphate buffered saline (PBS) stimulated equine neutrophils detected by LDCL (relative light units [RLU], mean ± SEM of six individual horses) over 90 min. Note peak of SOA generation in both ZAS and HIZAS stimulated cells is at 25 min post-stimulation and total abrogation of response occurs by 90 min.

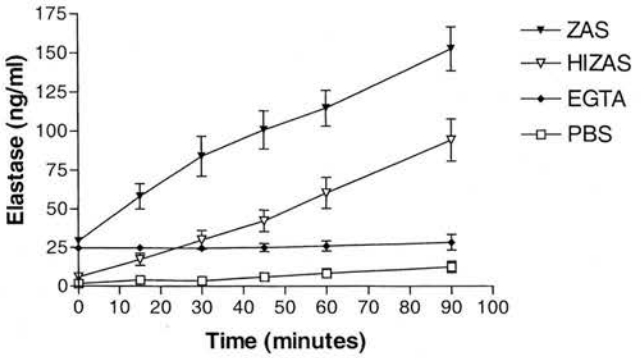

Fig. 1. Cumulative release of equine neutrophil elastase 2A (ENE 2A) from Percoll purified equine venous blood neutrophils stimulated with (ZAS), heat inactivated ZAS (HIZAS), ZAS + ethylene glycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and phosphate buffered saline (PBS), mean ± SEM of 6 individual horses. Note different characteristics of release pattern compared to superoxide anion (SOA) generation (Fig. 3) and the consistently higher concentration of ENE 2A in the ZAS + EGTA treated neutrophils compared to those treated with PBS.

stimulation with ZAS is significantly greater than that generated in the presence of HIZAS only over the first 90 min ($p = 0.03$–0.005). EGTA did not completely abrogate ENE 2A release in response to ZAS; there was no statistical difference between ZAS treated cells with or without EGTA in the amount of ENE 2A present in the supernatant at time zero ($p = 0.1$). The amount of ENE 2A released from neutrophils stimulated with ZAS + EGTA varies very little from the initial value at time zero suggesting that there is little or no further release of ENE 2A after this. Over the initial 90 min there is a significant difference between the amount of ENE 2A released from neutrophils treated with ZAS + EGTA and those treated with PBS ($p = 0.03$–0.005). By 120 min the 'leakage' of ENE 2A from cells treated with PBS had risen to a concentration whereby the difference between the two stimuli was no longer significant ($p = 0.07$).

Cell viability, as denoted by trypan blue exclusion, was 100% ($n = 6$ for each of the four stimuli) at time zero in all four stimuli. At 150 min post-stimulus mean cell viability in all cases was still > 99%.

4. Discussion

Equine neutrophils have been reported to contain about one third the NE found in human neutrophils (Dubin and Koj, 1986; Campbell et al., 1989) and this has been proposed as an explanation for emphysema being a relatively rare pathological change in the horse (Edelman et al., 1992). In this study, using an ELISA, we have shown the amount of ENE 2A per neutrophil to be over 0.8 pg/cell which is 100% greater than previous estimates derived by evaluation of enzyme activity (Dubin and Koj, 1986). In addition equine neutrophils contain an immunologically distinct elastase, elastase 2B (ENE 2B), (MW 20.5 kDa) at approximately a third the concentration and five times the elastinolytic activity of ENE 2A (Dubin et al., 1976), suggesting that the total amount of

ENE per equine neutrophil is even closer to the figure accepted for human neutrophils (1.1 pg/cell) (Campbell et al., 1989). The presence of cytosolic inhibitors of elastase in human and equine neutrophils means that elastase activity may be reduced during the extraction process when granular and cytosolic compartments are disrupted and their contents mixed. This implies that measurements of elastinolytic activity may underestimate the amount of elastase present. The present study supports this suggestion as a 55 kDa band was identified in horse neutrophil extracts (Fig. 1B) by the anti-ENE 2A antibody which had no demonstrable proteolytic activity (Fig. 1C) suggesting an elastase: inhibitor complex. The most likely candidate inhibitor in the horse neutrophil cytosol available to form this complex is horse leukocyte elastase inhibitor (HLEI) which has a MW of 35.2 kDa and is reported to be present at 0.5 pg/cell (Dubin, 1977; Dubin and Koj, 1986; Potempa et al., 1988). This study has also confirmed the presence of API in equine neutrophils at a low concentration (0.02 pg/cell) indicating that it may have a minor role to play compared to HLEI. This is probably analogous to human neutrophils which contain an abundant and probably major cytosolic inhibitor, SLPI, as well as lower levels of API (duBois et al., 1991; Sallenave et al., 1997).

While the release of elastase from human neutrophils has been investigated there is no similar data for the horse system. Previous studies have shown SOA generation and human NE release from human neutrophils to be variably dependent on extracellular divalent cations, the secretagogue type and concentration used (Khalfi et al., 1996). SOA generation from human neutrophils is dependent on extracellular calcium when the stimulus used is *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) but not when stimulated by phorbol-myristate-acetate (PMA) or opsonised zymosan (Khalfi et al., 1996). Elastase release from stimulated human neutrophils is more complicated. PMA stimulation is independent of calcium when the extracellular concentration ranges from 0 to 1.0 mM, but dose dependent from 1.5–4.0 mM; greater concentrations of calcium resulting in greater release of human NE (Khalfi et al., 1996). Conversely, FMLP induced human NE release is dependent upon extracellular calcium from 0 to 1.5 mM yet less affected at concentrations greater than 1.5 mM (Khalfi et al., 1996). In the current study the addition of EGTA abolished ZAS stimulated superoxide anion generation suggesting that this response is dependent on extracellular calcium and/or magnesium, typical of a receptor mediated, secondary messenger signalling mechanism (Korchak et al., 1984; Buhl et al., 1994) and similar to FMLP stimulated human neutrophils (Khalfi et al., 1996). However, ZAS-stimulated ENE 2A release from equine neutrophils appears to be only partially dependent upon extracellular calcium and/or magnesium. Immediately after the addition of ZAS the ENE 2A concentration in the supernatant was the same whether or not EGTA was present. This suggests a small degree of cation independence, utilisation of stored intracellular cations, or a significant difference in the kinetics of ZAS stimulation compared to chelation of cations by EGTA. This phenomenon requires further study as human neutrophils have been shown not to require a cytosolic calcium transient for degranulation or oxidative burst when stimulated by some receptor mediated stimuli (IgG, Seetoo et al., 1997). Another aspect requiring further investigation is the nature of the heat resistant components of ZAS as heat inactivation of ZAS did not completely remove its ability to stimulate neutrophil degranulation or SOA generation suggesting that there must be some stimulus present other than heat labile C5a.

The relationship between total SOA generation and total ENE 2A release during the initial 90 min post-ZAS stimulation (the duration of the oxidative burst) suggests that the extra-cellular release of the two products are closely linked, however, the kinetics of release are markedly different. Superoxide anions are generated and released in a respiratory burst lasting about 90 min, whereas elastase release although initiated at the start of the oxidative burst continues leading to a nearly linear increase in the extracellular concentration for at least 150 min post-ZAS stimulation. These findings indicate that while there may be a common signalling pathway initiating superoxide anion and elastase release (Roos, 1991), the post-initiation mechanisms of release are clearly different. Previous studies in other species have shown that superoxide anion generation by NADPH oxidase can become refractory despite continued presence of the stimulus (Rossi, 1986; Roos, 1991). However in these experiments elastase release does not appear to become refractory and this is something which requires further investigation as in vivo inflammatory stimuli may be present continuously for prolonged periods of time. In this study, on average, less than 10% of the total elastase content of equine neutrophils was released in the first 90 min post-stimulation. This suggests that either not all the ENE 2A present can be released under these conditions or, that there is sustained release that continues for some time after the respiratory burst.

In the healthy subject plasma derived API provides the main defence against elastase mediated tissue damage. Human API and the equine API isoform Spi1 are oxidation sensitive due to the presence of methionine residues at critical positions in the API molecules (Johnson and Travis, 1979; Matheson et al., 1982; Padrines et al., 1989; Patterson and Bell, 1989). One possibility that arises from the results of this study is that the simultaneous release of elastase and generation of SOA may allow for synergistic interaction. For example the early peak of superoxide anion release may act to down-regulate serpin inhibition of elastase activity in the extracellular micro-environment of the neutrophil by oxidising the sensitive API methionine residue. This would reduce the K_{ass} and K_i of the API/elastase interaction thereby reducing the effectiveness of inhibition (Cohen, 1979; Nakajima et al., 1979; Beatty et al., 1980; Travis et al., 1980; Hubbard et al., 1987; Potempa et al., 1991; Snider, 1992; Buhl et al., 1996). Potentially this mechanism would allow relatively uncontrolled proteolysis by elastase in the neutrophil's immediate environment resulting in tissue damage. This mechanism would be effective because as more neutrophils were recruited and activated at an on-going site of inflammation their oxidative bursts would act locally to overcome API inhibition. Recent data (Dagleish, unpublished observations), has shown that stimulating neutrophils in enriched media such as Iscove's modified Dulbecco's medium can increase the amount and duration of SOA generation. This implies that a nutrient rich environment, for example the extracellular tissue fluid present in vivo, may further enhance the respiratory burst resulting in greater potential for API inactivation than has been shown in the current in vitro studies.

The absence of panacinar emphysema in horses with COPD (Kaup et al., 1990), despite a pulmonary neutrophilia (Freeman et al., 1993; Dixon et al., 1995), a comparable NE content to human neutrophils and concurrent release of SOA and ENE 2A still needs to be addressed. Alpha-1-proteinase inhibitor is believed to be the most important protection against elastase activity at the alveolar level (Ohlsson, 1980; Hubbard et al.,

1989). As well as the oxidation sensitive isoform (Spi1) discussed above equine API also occurs as several abundant oxidation resistant isoforms (Spi2, 3 and occasionally 4) which are not found in humans (Patterson and Bell, 1989; Potempa et al., 1991). These isoforms may be resistant to SOA inactivation and therefore in the horse fully active API may always be present even in the presence of SOA generation whereas in human tissue all API present is oxidation sensitive. Alternatively equine neutrophils, despite pulmonary sequestration, may not be stimulated to degranulate fully in vivo, as is suggested by the low levels of release seen in the current study, resulting in a reduced proteolytic burden in equine pulmonary tissues.

5. Conclusion

This work shows that horse and human neutrophils have a similar elastase content to that reported in humans and thus species differences in the elastase content of neutrophils is not, as previously considered, responsible for observed differences in pulmonary emphysema patterns in humans and horses in neutrophil associated pulmonary disease. In addition ZAS stimulated equine neutrophils are wholly dependent on extracellular calcium and/or magnesium for SOA generation and highly dependent on extracellular cations for ENE 2A release. Further work is required to evaluate the release of ENE in response to other stimuli in vitro and to more clearly characterise the mechanisms involved. In addition the development of specific antibodies recognising equine API and elastase will make it possible to examine their relative content in pulmonary epithelial fluid lining and potential role in the pathogenesis of COPD.

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